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Under the Supervision of Professor Shilpa Buch

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TITLE

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Molecular mechanisms involved in neurotoxicity mediated by HIV proteins and drug abuse

Yu Cai, Ph.D.

University of Nebraska Medical Center, 2016

Supervisor: Shilpa Buch, Ph.D.

While the advancement of highly active antiretroviral therapy (HAART) has transformed the course of HIV/AIDS from a death sentence to a manageable chronic condition, the prevalence of a constellation of neurological disorders collectively termed as HIV-associated neurocognitive disorders (HAND) continues to persist in these patients. HAND is characterized by cognitive dysfunction, depression, impaired memory and/or deficits in motor skills. The underlying factors leading to HAND have been the subject of extensive research and are thought to be associated with HIV infection in the CNS combined with robust immune activation of resident cells of the CNS. In addition, there is a strong correlation between chronic substance abuse and the manifestation of HAND, posing a major challenge for health care management in HIV-positive drug abusers. One of the hallmark features of cocaine and opiate abuse is the increased neuronal toxicity in the setting of HIV infection.

Among the various commonly abused drugs, cocaine has been extensively studied for its ability to exacerbate the neuropathogenesis of HAND. Ample evidence suggests that cocaine not only facilitates viral replication in astrocytes and microglia, enhances the permeability of the blood-brain barrier (BBB) and exacerbates neuroinflammatory responses, working synergistically with viral proteins such as HIV Tat and virus envelop protein Gp120 to promote neuronal injury. Sigma-1 receptors (Sig-1R) are recognized as

a unique class of non-G protein-coupled intracellular protein that binds to their ligands such as cocaine, resulting in dissociation of Sig-1R from mitochondrion-associated ER membrane (MAM) to the endoplasmic reticulum (ER), plasma membrane, and nuclear membrane, regulating function of various proteins. Sig-1R has diverse roles in both physiological as well as in pathogenic processes. The disruption of Sig-1R pathways has been implicated as causative mechanism(s) in the development of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington Disease (HD). Additionally, the interaction of cocaine and Sig-1R has more recently been implicated in potentiating the pathogenesis of HAND through impairment of BBB, microglial activation and astrogliosis.

Opiates such as morphine are commonly used clinically in palliative care and pain management. Chronic exposure to morphine and other opioids however, can result in significant detrimental effects on cognition. Our findings suggest that morphine dysregulates synaptic balance in the hippocampus, a key center for learning and memory, via a novel signaling pathway involving reactive oxygen species (ROS), endoplasmic reticulum stress (ER stress) and autophagy. We demonstrate herein that morphine treatment leads to a reduction in excitatory and a concomitant enhancement of inhibitory synapse densities in the hippocampal neurons via activation of the mu opioid receptor. Furthermore, these effects are mediated by the ability of morphine to upregulate intracellular ROS from nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), which in turn, promotes ER stress and the induction of autophagy, leading ultimately to decreased excitatory and increased inhibitory synapse densities. The detrimental effects of morphine on synaptic densities were shown to be reversed by platelet-derived growth factor (PDGF), a pleiotropic growth factor, that has been implicated in neuroprotection, at the level of intracellular ROS. Both abnormal unfolded protein

response (UPR) and impaired autophagy have also been implicated as a causative mechanism in the development of various neurodegenerative diseases. The common underlying feature of most neurodegenerative diseases such as AD, prion diseases, PD, and ALS involves accumulation of misfolded proteins leading to initiation of endoplasmic ER stress and stimulation of the UPR. Additionally, ER stress has more recently been implicated in the pathogenesis of HAND. Autophagy plays an essential role in the clearance of aggregated toxic proteins and degradation of the damaged organelles. These results thus identify a novel cellular mechanism involved in morphine-mediated synaptic alterations with implications for impaired cognition as well as neurocognitive disorders. Furthermore, based on our findings, it can be speculated that therapeutic strategies aimed at activating PDGF signaling can be envisioned as possible approaches to block morphine-mediated cognitive decline.

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List of abbreviations

3-MA	3-Methyladenine
6-OHDA	6-Hydroxydopamine
AD	Alzheimer's disease
ADAD	autosomal-dominant Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
APP	amyloid beta (A4) precursor protein
ARHGAP26	Rho GTPase activating protein 26
ATF3	activating transcription factor 3
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
ATG12	autophagy related 12
ATG5	autophagy related 5
Aβ	β-amyloid
BBC3	BCL2 binding component 3
BCL2	B-cell CLL/lymphoma 2
BECN1	beclin1, autophagy related
C9orf72	chromosome 9 open reading frame 72

CALR	calreticulin
cART	combined anti-retrovirus therapy
CASP3	caspase 3, apoptosis-related cysteine peptidase
CBD	corticobasal degeneration
CHMP2B	charged multivesicular body protein 2B
CJD	Creutzfeldt-Jakob disease
CLU	clusterin
CSTB	cystatin B
CTSB	cathepsin B
DAPK1	death-associated protein kinase 1
DDIT3	DNA-damage-inducible transcript 3
DNAJ/HSP40	DnaJ (Hsp40) homolog
EHD1	EH-domain containing 1
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3
EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha
EIF2S1-P	phosphorylated eukaryotic translation initiation factor 2, subunit 1 alpha
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERN1	endoplasmic reticulum to nucleus signaling 1

fALS	familial amyotrophic lateral sclerosis
FOXO1	forkhead box O1
FTD	frontotemporal degeneration
FTLD	frontotemporal lobar degeneration
FUS/TLS	fused in sarcoma/translocated in liposarcoma
GRN	granulin
GSK3B	glycogen synthase kinase 3 beta
HAND	HIV-associated neurocognitive disorders
HD	Huntington disease
HSF1	heat shock transcription factor 1
HSP90	heat shock 90 kDa protein
HSP90B	heat shock protein 90kDa beta
HSPA	heat shock 70 kDa protein
HSPA5	heat shock 70kDa protein 5
HTT	huntingtin
IL1B	interleukin 1, beta
ITPR	inositol-1,4,5-trisphosphate receptor
MAP1LC3B	microtubule-associated protein 1 light chain 3 beta
MAPK8	mitogen-activated protein kinase 8
MAPT/tau	microtubule-associated protein tau

MBTPS1	membrane-bound transcription factor peptidase, site 1
MBTPS2	membrane-bound transcription factor peptidase, site 2
MICALL1	MICAL-like 1
mOPTN	mutant optineurin
MPP+	1-methyl-4-phenyl-pyridinium
mPSEN1	mutant presenilin-1
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mSOD1	mutant superoxide dismutase 1
MTORC1	mechanistic target of rapamycin [serine/threonine kinase] complex1
NLRP3	NLR family, pyrin domain containing 3
OPTN	Optineurin
PARK2	parkin RBR E3 ubiquitin protein ligase
PARK7	parkinson protein 7
PART	primary age-related taupathy
PAS	pre-autophagosomal structures
PD	Parkinson's disease
PDI	protein disulfide isomerase
PDIA3	protein disulfide isomerase family A, member 3
PE	phosphatidylethanolamine

PINK1	PTEN induced putative kinase 1
PPP1	protein phosphatase 1
PPP1R15A	protein phosphatase 1, regulatory subunit 15A
PRNP	prion protein
PSEN1	presenilin-1
PSEN2	presenilin-2
PSP	progressive supranuclear palsy
PtdIns3K	class III lipid kinase
RTN3	reticulon 3
RYR	ryanodine receptor
sALS	sporadic amyotrophic lateral sclerosis
SIRT1	sirtuin 1
SNCA/α-synuclein	synuclein, alpha
SNpc	substantia nigra pars compacta
SQSTM1	sequestosome 1
STIP1	stress-induced phosphoprotein 1
TARDBP	TAR DNA binding protein
Tat	HIV-1 Transactivator of transcription
TBI	traumatic brain injury
TTR	transthyretin

UBQLN1   ubiquilin-1

UBQLN2   ubiquilin-2

ULK1   unc-51 like autophagy activating kinase 1

UPR   unfolded protein response

VCP   valosin-containing protein

XBP1   X-box-binding protein 1

XBP1s   spliced form of X-box-binding protein 1

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CHAPTER I

INTRODUCTION

1. REGULATION OF MORPHINE INDUCED SYNAPTIC ALTERATIONS

1.1 Overview

Opioids such as morphine are the most potent and efficacious drugs currently available for the management of moderate to severe acute and chronic pain (Fields 2011). While these drugs are commonly prescribed in clinical practice for their beneficial analgesic effects, abuse of opioids such as morphine, often results in deleterious side effects such as opioid tolerance, dependence, addiction and detrimental effects on cognitive performance (Kurita, Sjogren et al. 2011, Kadivar, Farahmandfar et al. 2014, Farahmandfar, Kadivar et al. 2015, Fields and Margolis 2015). Emerging evidence suggests that neural circuits associated with addiction share some common pathways with those underlying learning and memory (Nestler 2002, Kelley 2004, Hyman, Malenka et al. 2006). It has been well documented that morphine interferes with learning and memory processes in both healthy individuals as well as in cancer patients (Kerr, Hill et al. 1991, Hill and Zacny 2000, Skaer 2004, Bodnar and Klein 2005, Kurita, Sjogren et al. 2011). Identifying molecular aberrations underlying morphine abuse that lead to cognitive changes thereby providing avenues for reversing these changes, remains a pressing need in the field.

The hippocampus, an integrative center for regulating learning and memory and addiction (Koob and Volkow 2010), is also one of the brain regions with significant expression of mu opioid receptor (MOR) (Le Merrer, Becker et al. 2009), suggesting that the effects of opioids on cognition could be mediated by signaling via the MOR in the hippocampus. Alterations in synaptic density, structure, function and plasticity have been identified as key components of the finely tuned machinery underlying cognition in the hippocampus (Marder and Goaillard 2006, Zhong and Gerges 2010). Such mechanisms

operate at both excitatory and inhibitory synapses (Bateup, Johnson et al. 2013, Calfa, Li et al. 2015, Nelson and Valakh 2015, Villa, Berry et al. 2016).

1.2 Morphine-mediated dysregulation of synapses

Both *in vitro* and *in vivo* studies have demonstrated that morphine regulates excitatory spine density in the hippocampus (Robinson, Gorny et al. 2002, Liao, Lin et al. 2005, Liao, Grigoriants et al. 2007, Zheng, Zeng et al. 2010). A profound loss in dendritic spines in the hippocampal CA1 was observed in rats exposed to long-term administration of morphine (Robinson, Gorny et al. 2002). Consistent with this finding, studies also indicate a reduction in spine density in cultured hippocampal neurons treated with morphine *in vitro* (Liao, Lin et al. 2005, Liao, Grigoriants et al. 2007, Zheng, Zeng et al. 2010). However, our knowledge of the molecular mechanisms that regulate the ability of morphine to regulate spines and excitatory synapses remains far from complete. The effects of morphine on inhibitory synapses remain unclear. The balance of excitatory and inhibitory synapses is crucial for maintenance of normal functioning of neural circuits (Froemke, Merzenich et al. 2007) and higher order brain functions including cognition. There are limited avenues for therapeutic intervention to address cognitive deficits resulting from long-term use of morphine. Identifying molecular mechanisms underlying morphine-mediated synaptic alterations is thus of critical importance in our understanding of how neural circuitry responds to opioids and could provide insights into the development of therapeutic targets aimed at restoring normal cognitive function in morphine users.

1.3 Role of ROS-ER Stress-Autophagy axis in morphine-mediated synaptic alterations

We have identified a key signaling pathway that allows morphine signaling via the MOR and its downstream ROS-ER Stress-Autophagy axis to regulate both excitatory and inhibitory synaptic density in the hippocampus **(Figure 1)**.
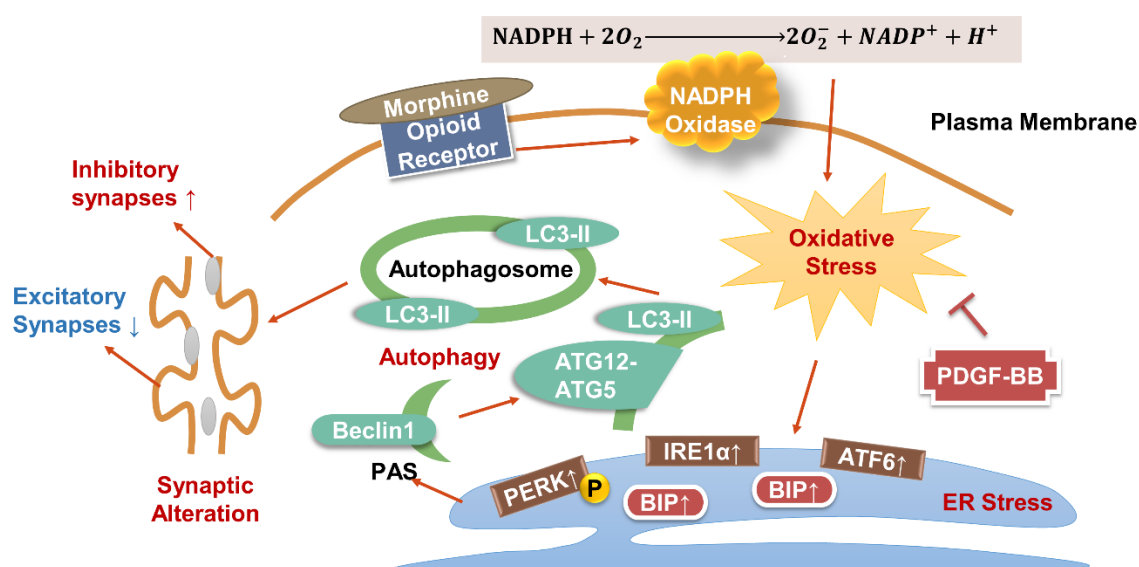
Figure 1 Schematic of morphine-mediated synaptic alterations.

Synaptic alterations induced by morphine involve a novel mechanism that requires sequential activation of MOR, oxidative stress, ER stress and autophagy. PDGF-BB ameliorates morphine-mediated synaptic alterations at the level of ROS generation and its downstream signaling.

Oxidative stress is the imbalanced state of reactive oxygen species (ROS) generation and elimination that has been reported in various neurodegenerative disorders (Andersen 2004). The highly metabolic nature of the brain with high oxygen consumption results in the robust production of ROS in neurons. The levels of ROS are tightly maintained through the activity of a network of antioxidants. At physiologically relevant concentrations, ROS have been demonstrated to participate in cellular mechanisms that underlie synaptic plasticity and hence cognitive functions (Massaad and Klann 2011). Excessive levels of ROS are also associated with decreases in cognitive performance. The ability of morphine to elicit alterations in the level of ROS is consistent with data from other cells such as human neuroblastoma, murine macrophages and microglia (Bhat, Bhaskaran et al. 2004, Ma, Yuan et al. 2015, Cai, Kong et al. 2016). Excessive ROS generation affects synaptic plasticity and interferes with the cognitive functioning (Massaad and Klann 2011). Two major sources of ROS production in the brain are mitochondria and membrane-bound NADPH oxidase. NADPH oxidase plays an essential role in neuro-inflammation in various neurodegenerative disorders such as HIV-associated neurocognititve disorders (HAND) (Williams, Yao et al. 2010). Recent emerging evidence suggests that NADPH oxidase inflicts oxidative damage on synaptic function under ischemic/reperfusion injury (Murotomi, Takagi et al. 2011, Beske, Byrnes et al. 2014).

The endoplasmic reticulum is a subcellular compartment that governs protein quality control in the secretory pathway to prevent protein aggregation and misfolding (Cai, Arikkath et al. 2016). Production of ROS also affects ER homeostasis and protein folding, leading to aberrant aggregation of misfolded proteins that termed as ER stress (Malhotra and Kaufman 2007). Activation of ER stress promotes the unfolded protein response (UPR) to reestablish homoeostasis. UPR is an adaptive mechanism initiated by dissociation of

ER chaperone BIP from major ER stress sensors pPERK, IRE1α and ATF6 in the presence of ER stress (Roussel, Kruppa et al. 2013). While the role of these pathways in relation to neurodegenerative disorders has been explored (Hetz and Mollereau 2014), it remains unclear how ER stress that does not lead to cell death contributes to other neuronal processes including neuro-inflammation (Guo, Liao et al. 2015), synaptic failure and neuronal loss (Moreno, Radford et al. 2012). Although ER stress (Huang, Hu et al. 2015) and components of the UPR response have been linked to deficits in learning and memory (Martinez, Vidal et al. 2016), the underlying mechanisms remain incompletely understood.

Autophagy is an intracellular process that delivers cellular organelles and misfolded proteins from the autophagosomes to the lysosomes for degradation (Zhang and Baehrecke 2015), another cellular defense mechanism against ER stress (Ogata, Hino et al. 2006). Substances of abuse such as cocaine and morphine have been shown to induce autophagy in the human neuroblastoma cell line SH-SY5Y and rat hippocampus (Zhao, Zhu et al. 2010) and microglia (Guo, Liao et al. 2015). It is worth noting that previous study has indicated that neuronal autophagy enables the process of synaptic pruning (Tang, Gudsnuk et al. 2014), suggesting the possibility that autophagy could be involved in morphine-mediated synaptic injury.

Our data indicate that morphine mediated activation of the MOR results in the generation of ROS leading to downstream effects including activation of ER stress pathways followed by initiation of the autophagy cascade. This eventually results in a decrease in excitatory synapse density accompanied by an increase in the density of inhibitory synapses, which likely contributes to the cognitive decline associated with morphine use.

1.4 Neuroprotective role of Platelet-derived growth factor-BB (PDGF-BB)

The detrimental effects of morphine on the density of both excitatory and inhibitory synapses can be ameliorated by platelet-derived growth factor-BB (PDGF-BB). Platelet-derived growth factor-BB (PDGF-BB) is a pleiotropic protein with crucial roles in various cellular processes during both developmental stages and pathogenesis. Our lab has demonstrated PDGF-BB exerts neuroprotective effects against HIV-associated neurocognitive disorders (HANDs) and regulates synaptic plasticity gene Arc/Arg3.1 (Peng, Dhillon et al. 2008, Peng, Yao et al. 2010), but whether PDGF also protects hippocampal neurons from morphine-mediated synaptic injury remains elusive. Members of the platelet-derived growth factor (PDGF) family not only regulate neural development but also function as broad-acting neuroprotective agents against various oxidative and excitatory insults (Smits, Kato et al. 1991, Cheng and Mattson 1995, Tseng and Dichter 2005, Tang, Arjunan et al. 2010, Lee, Zhang et al. 2013). These studies thus provide a key mechanistic link between morphine use and cognitive decline and offer a potential avenue for preventing or perhaps reversing these cognitive deficits.

2. Interplay of Endoplasmic Reticulum Stress and Autophagy in Neurodegenerative Disorders

2.1 Overview

Neurodegenerative disorders are characterized by progressive loss of neuronal functions that are closely associated with loss/dysfunction of neuronal cells. Emerging evidence suggests that accumulation of exogenous or abnormal misfolded proteins leads to a state of endoplasmic reticulum (ER) stress in neurons, contributing to the salient pathology associated with neurodegeneration. In order to restore homeostasis within the ER there occurs sequential activation of the unfolded protein response (UPR) involving translational attenuation of global protein synthesis, transcriptional induction of genes functioning as ER chaperones, and the ER-associated degradation (ERAD) of aggregated proteins ultimately leading to autophagy (Matus, Lisbona et al. 2008, Buchberger, Bukau et al. 2010). Autophagy is an essential catabolic mechanism that delivers misfolded proteins and damaged organelles to the lysosome for degradation (Mizushima and Komatsu 2011). Maintaining basal levels of autophagic activity is critical for postmitotic neurons that are unable to dispose of aggregated proteins via cell division (Son, Shim et al. 2012). As a result, increased dependence of neurons on a higher threshold of constitutive, basal levels of autophagy thus renders these cells more vulnerable to impairments in autophagy, a key feature underlying most neurodegenerative diseases. Accumulating evidence suggests that in chronic neurodegenerative disorders, persistent ER stress often results in stimulation of autophagic activities, likely as a compensatory mechanism, to relieve ER stress; however, in the face of impaired UPR or autophagy, there is inefficient clearance of the accumulated proteins, which in turn, leads to the development and progression of neurodegeneration. This review is an attempt to provide a survey of the major ER stress signaling pathways and the autophagic mechanism(s)

and their interplay in the development and progression of neurodegenerative diseases such as AD, prion diseases, PD, ALS and HAND.

2.2 ER stress and UPR

ER stress is a pathological state that involves the aberrant aggregation of misfolded proteins, which in turn, results in the UPR that is initially expected to rescue cells from the stress and restore homeostasis within the ER (Buchberger, Bukau et al. 2010). The ER stress stimuli in neurodegenerative disorders include accumulation of exogenous viral proteins (Norman, Perry et al. 2008), aggregation of mutant endogenous proteins (Atkin, Farg et al. 2008) and depletion of ER calcium stores via ITPR (inositol-1,4,5-trisphosphate receptor) (Haughey, Holden et al. 1999, Leissring, LaFerla et al. 2001) and RYR (ryanodine receptor) (Norman, Perry et al. 2008). Chaperones within the ER, such as HSPA5 (heat shock 70kDa protein 5 [glucose-regulated protein, 78kDa]), and CALR (calreticulin), require high levels of $[Ca^{2+}]_{ER}$ for binding to paired anionic amino acids to carry out the function of protein folding **(Figure 2.1)** (Lucero and Kaminer 1999, Ma and Hendershot 2004). Thus, depletion of ER calcium store renders ER-resident chaperones inactive for proper protein folding processes, resulting in the accumulation of misfolded proteins.

In the central nervous system, the UPR is initiated by 3 major cascading transmembrane sensors: a) EIF2AK3 (eukaryotic translation initiation factor 2-alpha kinase 3), b) ERN1 (endoplasmic reticulum to nucleus signaling 1) and c) ATF6 (activating transcription factor 6) (Kaufman 2002). The most abundant ER chaperone, HSPA5 plays an essential role in initiation of the UPR by 3 major sensors (Kaufman 2002). The ER-luminal domain of EIF2AK3, ERN1 and ATF6 interacts with HSPA5 to suppress ER stress

under physiological conditions **(Figure 2.1).** Following cellular stress and accumulation of

unfolded proteins, there is dissociation of HSPA5 from the ER sensor proteins, leading to

the activation of ER stress sensors (Schroder and Kaufman 2005).
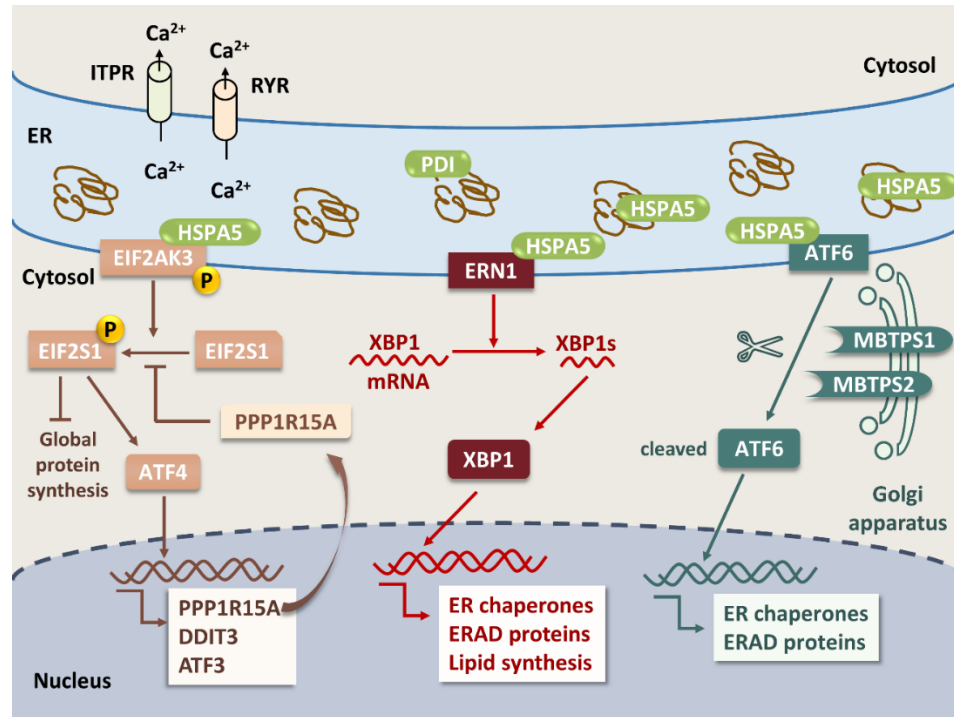
Figure 2.1 ER stress and UPR pathways in neuronal cells.

Pathological accumulation of misfolded proteins and/or depletion of ER calcium store via activation of ITPR and RYR leads to ER stress. Dissociation of HSPA5 from 3 ER stress sensors, EIF2AK3, ERN1 and ATF6, results in phosphorylation of EIF2AK3 and ERN1 and translocation of ATF6 to the Golgi apparatus. Activated EIF2AK3 is a serine/threonine protein kinase that phosphorylates EIF2S1. p-EIF2S1 (phosphorylated EIF2S1) inhibits global protein synthesis but selectively upregulates ATF4, PPP1R15A, DDIT3 and ATF3. PPP1R15A provides a negative feedback by dephosphorylating p-EIF2S1. ERN1 cleaves *XBP1* mRNA; the spliced form of *XBP1* encodes the XBP1 protein. XBP1 increases the expression of genes encoding ER chaperones, ERAD proteins and lipid synthesis to restore the capacity of protein folding. ATF6 is translocated to the Golgi apparatus where it is cleaved by MBTPS1 and MBTPS2, and cleaved ATF6 stimulates the expression of ER chaperones and ERAD proteins. Apoptosis will ensue if upregulation of ER chaperones and ERAD proteins fails to rescue ER stress.

EIF2AK3 is a serine/threonine protein kinase that is activated by dimerization and trans-autophosphorylation following dissociation from HSPA5 **(Figure 2.1)** (Harding, Zhang et al. 1999). Phosphorylation of EIF2S1 (eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa) mediated by active EIF2AK3 causes a shift in the open reading frames from the true translation initiation site to a site upstream of the coding region of ATF4 (activating transcription factor 4) as well as ATF3 (activating transcription factor 3), DDIT3 (pro-apoptotic transcription factor, DNA-damage-inducible transcript 3) and PPP1R15A (protein phosphatase 1, regulatory subunit 15A), a cofactor for PPP1 (protein phosphatase 1) that serves as a negative-feedback regulator of EIF2S1 phosphorylation (Lu, Harding et al. 2004, Hotamisligil 2010). Activation of EIF2AK3 therefore attenuates global translation of proteins while paradoxically stimulating the expression of stress response genes. However, if the UPR fails to rescue neurons from ER stress, ensuing ER stress culminates in activation of apoptosis (Paschen and Doutheil 1999).

ERN1 is a ubiquitous serine/threonine kinase **(Figure 2.1)**. Similar to EIF2AK3, following dissociation of HSPA5, the kinase domain of ERN1 is activated. However, unlike EIF2AK3, ERN1 contains a C-terminal endoribonuclease domain that is also activated following accumulation of the unfolded proteins (Calfon, Zeng et al. 2002). The endoribonuclease domain is responsible for the splicing of the mRNA encoding the transcriptional factor XBP1 (X-box binding protein 1) (Calfon, Zeng et al. 2002). The protein XBP1 translated from *XBP1s* (spliced form of *XBP1* mRNA) in turn stimulates the expression of ER chaperones, ERAD proteins and lipid synthesis to restore protein folding capacity within the ER (Ron and Walter 2007)**.**

ATF6 is also an ER stress transmembrane sensor belonging to a family of bZIP transcription factors **(Figure 2.1)** (Hetz and Glimcher 2009). In response to the UPR, HSPA5 dissociation releases ATF6 from the ER membrane allowing it to translocate to

the Golgi, where it is cleaved by proteolytic cleavage to a soluble form resulting in its trafficking into the nucleus (Haze, Yoshida et al. 1999, Chen, Shen et al. 2002, Sato, Nadanaka et al. 2011). Following translocation into the nucleus, ATF6 functions as a transcription factor responsible for recovery from acute stress with tolerance to chronic stress by inducing the expression of ER chaperone genes such as those encoding HSPA5 and PDI (protein disulfide isomerase) folding enzymes and the ERAD components (Wu, Rutkowski et al. 2007).

Overall, the activation of ER stress leads to attenuation of global protein synthesis, induction of ER chaperones to increase the capacity of the ER for protein folding and induction of ERAD elements to decrease the ER protein load (Ron and Walter 2007).

2.3 Autophagy

Macroautophagy (hereafter autophagy) is a tightly regulated catabolic pathway for lysosomal degradation of cytoplasmic organelles or cytosolic components and the recycling of the resulting macromolecules (Wong and Cuervo 2010). Homeostasis of autophagic activities in the cytoplasm is critical for maintenance of neuronal functioning. There is increasing evidence that failure in clearing the aggregated proteins or impaired organelles contribute to programmed cell death or apoptosis (Green and Levine 2014). Intriguingly, abnormal autophagic activity has been described in AD (Nixon, Wegiel et al. 2005), HD (Kegel, Kim et al. 2000), PD (Anglade, Vyas et al. 1997) and Creutzfeldt-Jakob disease (CJD) (Sikorska, Liberski et al. 2004).

Autophagy is initiated by the sequestration of targeted substrates within double-membrane vesicles termed phagophores that mature into autophagosomes (Xie and

Klionsky 2007). Prior to autophagosome fusion with the lysosome and degradation of the sequestered cargo, autophagy undergoes a series of key steps including activation by signal transduction, phagophore nucleation, membrane elongation, lysosomal fusion and cargo degradation **(Figure 2.2)** (Liang 2010).
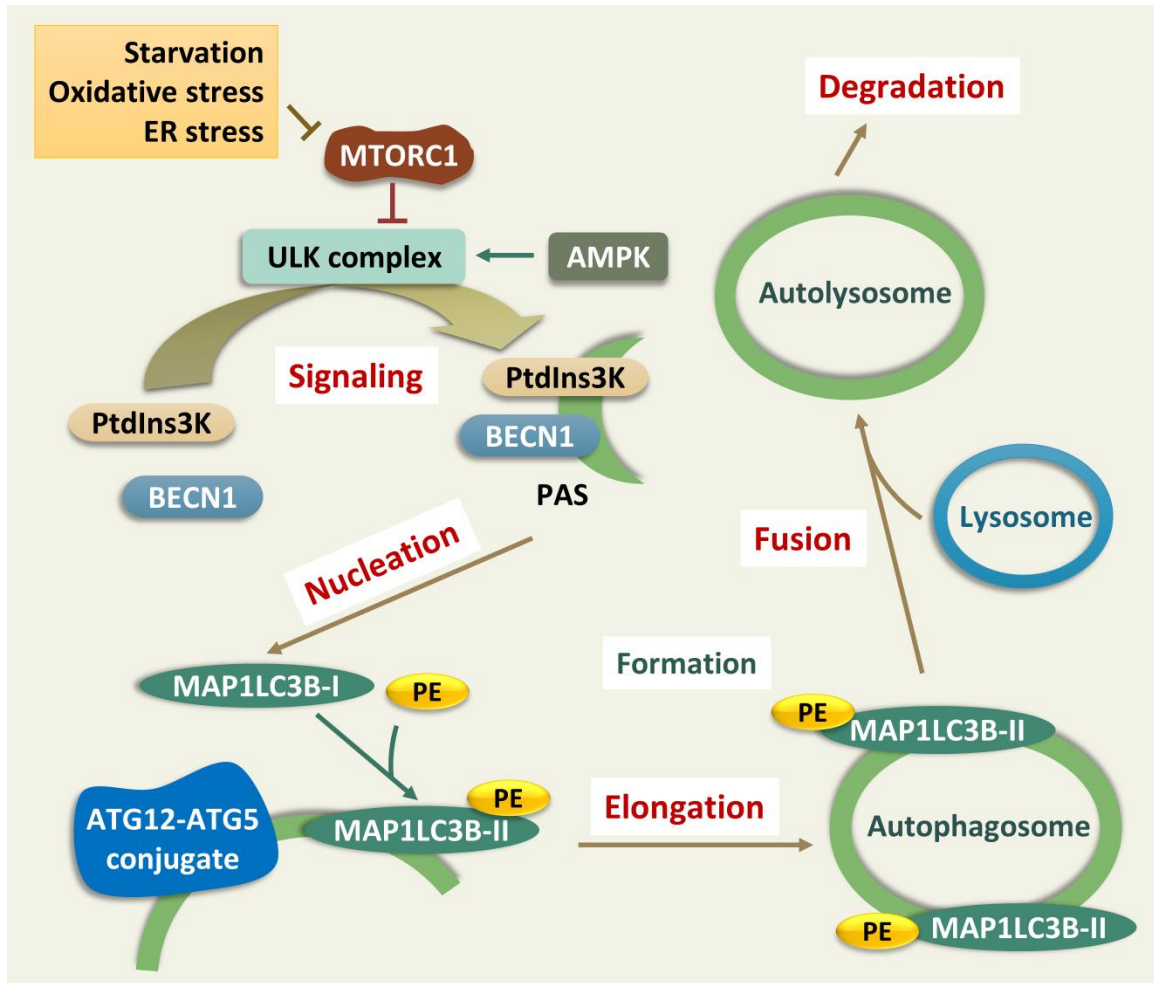
Figure 2.2 The autophagic cascade in neurodegenerative disorders.

Stress stimuli associated with neurodegenerative disorders inhibits MTORC1, resulting in activation of the ULK1 complex. The ULK1 complex can also be activated by AMPK. The ULK1 complex initiates vesicle nucleation by translocating BECN1 and PtdIns3K to phagophores. To elongate the membrane from the phagophore assembly site (PAS), The ATG12–ATG5 conjugate regulates the conjugation of PE to MAP1LC3B, resulting in conversion of MAP1LC3B from soluble MAP1LC3B-I into phagophore and autophagosome-associated MAP1LC3B-II. Once the autophagosome is formed, it fuses with a lysosome for degradation of its sequestered cargo.

Stress stimuli, such as starvation, oxidative stress and ER stress, result in inhibition of MTORC1 (mechanistic target of rapamycin [serine/threonine kinase] complex 1) and, reciprocally, activation of AMPK (AMP-activated protein kinase) (Kroemer, Marino et al. 2010), both of which lead to activation of the ULK1 (unc-51 like autophagy activating kinase 1) complex by changing the phosphorylation state of certain components in the complex (Mizushima 2010). The activated ULK1 complex initiates vesicle nucleation by translocating the BECN1 (beclin 1, autophagy related)-containing core multiprotein complex and the class III phosphatidylinositol 3-kinase from the cytoskeleton to sites of phagophore assembly (Suzuki, Kubota et al. 2007, Sinha and Levine 2008, Di Bartolomeo, Corazzari et al. 2010, Mizushima 2010). Vesicle nucleation is followed by membrane elongation. In the process of elongation of autophagic membrane, ATG12 (autophagy related 12) conjugated to ATG5 (autophagy related 5) positively regulates the conjugation of phosphatidylethanolamine to MAP1LC3B (microtubule-associated protein 1 light chain 3 beta) (Ichimura, Kirisako et al. 2000, Nixon 2013) followed by the conversion of MAP1LC3B from soluble MAP1LC3B-I into autophagic vesicle-associated MAP1LC3B-II (Yang and Klionsky 2010). MAP1LC3B-II stably binds to the phagophore and autophagosome membranes (Tanida, Ueno et al. 2008). Finally, the autophagosomes fuse with the lysosome for degradation of the sequestered cargo (He and Klionsky 2009). Moreover, endocytic recycling is a complicated process correlated with endocytic uptake to regulate the components of the plasma membrane. Regulation of endocytic recycling involves various molecules such as RAB1A, RAB11, RAB8, RAB22, ARHGAP26 (Rho GTPase activating protein 26), EHD1 (EH-domain containing 1), and MICALL1 (MICAL-like 1) (Hattula, Furuhjelm et al. 2006, Grant and Donaldson 2009, Maldonado-Baez and Donaldson 2013, Cai, Xie et al. 2014, Cai, Xie et al. 2014, Xie, Naslavsky et al. 2014). Recent evidence also demonstrates that recycling endosomes promote autophagy (Longatti, Lamb et al. 2012).

Autophagy is highly conserved in all types of eukaryotic cells and is essential for the regulation of basic cellular processes such as growth and apoptosis. Among all the other cell types, neurons exhibit higher basal levels of autophagy. This could likely be attributed to their structure and function: (1) Neurons are the largest cells in the human body (based on their length) and have highly specialized structures such as axons and dendrites that are the sites for synthesis of macromolecules including proteins, RNA and lipids. Appropriate delivery and degradation of these macromolecules is essential for homeostatic maintenance of normal synaptic growth and activity. (2) Neurons are non-dividing cells that are extremely sensitive to accumulation of toxic aggregates compared to the other mitotic cells. (3) Neurons require higher energy for execution of their normal functions. In summary, owing to the increased susceptibility of neurons to genetic/environmental insults, they are endowed with tightly regulated and effective intrinsic mechanisms such as autophagy, to execute their normal functions during a pathological insult. Protein quality control by autophagy is thus an essential attribute for neuronal survival and functioning. Dysfunction of the autophagic activities impedes synaptic development (Tang, Gudsnuk et al. 2014), hampers axonal function and could underlie the onset and progression of various neurodegenerative disorders (Son, Shim et al. 2012). Paradoxically, however, once the UPR and autophagy responses are unable to eliminate the damaged proteins or organelles effectively, neurons become highly susceptible to the diseases of protein aggregation underlying the neurodegenerative diseases.

2.4 ER stress and autophagy

Emerging studies also demonstrated a mechanistic link between the ER stress and autophagy, in which the activation of EIF2AK3 is essentially required due to the misfolded protein accumulation shown in various diseases to induce autophagy (Kouroku, Fujita et al. 2007, Adolph, Tomczak et al. 2013). Furthermore, the UPR downstream mediators of EIF2AK3 such as ATF4 and DDIT3 were also reported to guide the induction of autophagy gene transcription such as with *BECN1*, *MAP1LC3B*, *ATG5*, *ATG7* and *ATG12* in response to amino acid starvation or ER stress (B'Chir, Maurin et al. 2013). Hypoxic microenvironments also activate EIF2AK3- and ATF4-DDIT3 signaling, which is ultimately involved in the regulation of MAP1LC3B and ATG5 proteins to induce autophagy (Rouschop, van den Beucken et al. 2010, Rzymski, Milani et al. 2010). Another arm of ER stress, involving ERN1, is also involved in autophagy induction via activating AMPK in response to nitric oxide (Meares, Hughes et al. 2011). Alternatively, ERN1 induces autophagy through dissociation of BECN1 from its binding with the anti-apoptotic protein BCL2 (B-cell CLL/lymphoma 2)(Wei, Pattingre et al. 2008, Deegan, Saveljeva et al. 2013) via MAPK8 (mitogen-activated protein kinase 8)-mediated phosphorylation of BCL2 (Urano, Wang et al. 2000, Haberzettl and Hill 2013). Factors downstream of ERN1, such as XBP1, also play an essential role through an interaction with FOXO1 (forkhead box O1) in the negative feedback loop of ER stress-mediated autophagy (Zhou, Lee et al. 2011, Vidal, Figueroa et al. 2012, Zhao, Li et al. 2013). In addition, the soluble ATF6 formed after proteolytic cleavage under ER stress can upregulate the expression level of DAPK1 (death-associated protein kinase 1) (Kalvakolanu and Gade 2012, Gade, Manjegowda et al. 2014). The increased DAPK1 phosphorylates BECN1 leading to the effective dissociation from its negative regulator BCL2, thereby triggering autophagy **(Figure 2.3)** (Zalckvar, Berissi et al. 2009). Overall, the misfolded/aggregated proteins induce ER

stress and shackle the autophagy functions in various diseases including neurodegenerative diseases.
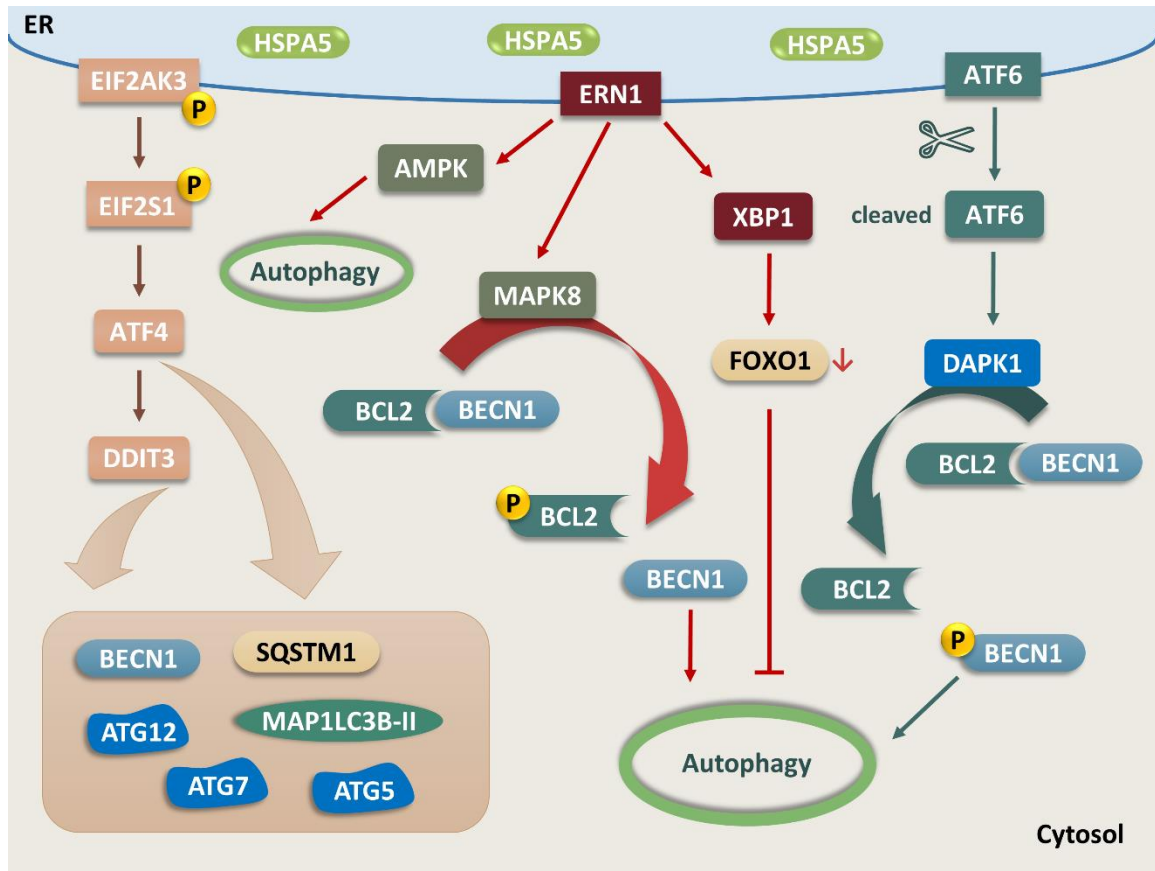
Figure 2.3 The interplay between the UPR and autophagy.

EIF2AK3-EIF2S1 signaling induces the expression of various autophagy-related proteins via ATF4 and DDIT3. ERN1 triggers autophagy through activation of AMPK and dissociation of BECN1 from BCL2 via MAPK8-dependent phosphorylation of BCL2. Conversely, XBP1 that acts as the downstream mediator of ERN1 provides negative feedback for autophagy by promoting degradation of FOXO1. Upregulation of DAPK1 induced by cleaved ATF6 phosphorylates BECN1 and leads to dissociation of BECN1 from BCL2, resulting in enhanced autophagic activities.

Intracellular protein aggregation is one of the salient features underlying various neurological diseases such as AD and PD (Pollanen, Dickson et al. 1993, Price, Tanzi et al. 1998). The balance between protein generation and degradation is crucial for protein homeostasis. Intriguingly, while most of the aggregated proteins result in the common endpoint of neuronal impairment, the mechanism of action of each of the aggregated proteins is distinct. For instance, aggregated $A\beta$ (β-amyloid) that is cleaved from its membrane-bound precursor APP (amyloid precursor protein), is a primary source of ER stress in AD (Suen, Lin et al. 2003, Alberdi, Wyssenbach et al. 2013), whereas in PD dysfunction appears to result from the accumulation of Lewy bodies and intracellular inclusion bodies containing diffuse deposits of misfolded SNCA/α-synuclein (synuclein, alpha [non A4 component of amyloid precursor])(Pollanen, Dickson et al. 1993). ER stress is likely a secondary response due to oxidative stress and/or inflammation mediated by the aggregates (Hashimoto, Hsu et al. 1999, Frank-Cannon, Tran et al. 2008, Gao, Zhang et al. 2011, Imaizumi, Okada et al. 2012). Taken together, based on the nature of the disease the sequence of events mediated by the aggregated proteins can be very different. For example, in the case of AD, $A\beta$ is the primary source of ER stress whereas in PD, the accumulated SNCA first induces cellular responses, which, in turn mediate the secondary ER stress leading to disease pathogenesis.

Similar to the diversity of signaling events observed in AD and PD, differences in the tissue responses in the accumulation of aggregated proteins in the autosomal dominant mutations in PD and AD also show striking differences. Genetic mutations (accounting for autosomal-dominant AD) in APP, PSEN1 (presenilin 1) or PSEN2 (presenilin 2) divert the normal processing of APP towards the amyloidogenic phenotype, leading in turn to enhanced production and deposition of total $A\beta$ and $A\beta$ peptides, that have a greater tendency to aggregate and mediate disease pathogenesis (Suarez-Calvet,

Belbin et al. 2014). Unlike AD wherein augmented production of aggregated proteins plays a major role in disease pathogenesis, in the autosomal dominant mutations of PD such as SNCA and LRRK2 (leucine-rich repeat kinase 2), protein degradation pathways are impeded, leading to accumulation of faulty aggregated proteins. It has been well documented that mutant SNCA impedes autophagy by inhibiting the GTPase RAB1A, whereas mutations in LRRK2 hinder the autolysosomal degradation pathways in the late stage of autophagic activity (Gomez-Suaga, Churchill et al. 2012, Lynch-Day, Mao et al. 2012). Intriguingly therefore while accumulation of aggregated protein response underlies both the autosomal dominant mutant form of AD and PD, the mechanism(s) by which the protein aggregation occurs (impaired protein degradation in PD versus increased protein production in AD) leading to disease pathogenesis, is unique for each disease. A better understanding of the tissue response in mediating the aberrant protein aggregation is thus critical in dissecting the mechanism(s) underlying disease pathogenesis and for future development of therapeutic targets.

2.5 Alzheimer's disease and other tauopathies

AD, one of the most common neurodegenerative diseases in the elderly, is characterized clinically as an ongoing cognitive impairment with typical pathogenic deposits of neurofibrillary tangles containing hyperphosphorylated MAPT/tau (microtubule-associated protein tau) and extracellular accumulations of Aβ plaques (Selkoe 2001). Aβ is the cleaved product released from its precursor protein, APP, following exposure to BACE1/β-secretase and γ-secretase (Chen, Gamache et al. 2014). The complex of γ-secretase, composed of at least PSEN1 and PSEN2 in the ER, cleaves APP to Aβ of varying lengths—$A\beta_{40}$ and $A\beta_{42}$. Genetic mutations in *APP*, *PSEN1* and

PSEN2 account for autosomal-dominant AD (Agyemang, Harrison et al. 2015). Another salient feature of AD is pathological aggregation of the MAPT protein (Nijholt, van Haastert et al. 2012). MAPT is an axonal cytosolic protein for the stabilization of the microtubule network, but it becomes hyperphosphorylated and aggregated within neurons in the form of neurofibrillary tangles (Nijholt, van Haastert et al. 2012).

UPR and autophagy play an important role in protecting neurons against accumulation of Aβ and PSEN1 mutations in the familial form of AD. PSEN1 mutations and intracellular Aβ, but not extracellular Aβ, have been reported to trigger ER stress in neurons by releasing ER calcium into the cytosol via ITPR and RYR (Suen, Lin et al. 2003, Briggs, Schneider et al. 2013, D'Adamio and Castillo 2013). Similar to neurons, Aβ also triggers calcium-dependent ER stress in glial cells, which is indicated to play an important role in Aβ-mediated astrogliosis *in vivo* (Alberdi, Wyssenbach et al. 2013). Interestingly, although Aβ mutation upregulates the expression level of HSPA5 in neurons, HSPA5 plays a neuroprotective role against ER stress-associated cell death by suppressing oxidative stress and maintaining calcium homeostasis (Yu, Luo et al. 1999). Intriguingly, unlike Aβ, mutations of PSEN1 inhibit major ER stress sensors including HSPA5, EIF2AK3, ERN1 and ATF6, rendering neurons vulnerable to ER stress stimuli (Katayama, Imaizumi et al. 1999, Katayama, Imaizumi et al. 2004). Thus, the UPR plays a neuroprotective role in AD but can be disrupted by either accumulation of Aβ or PSEN1 mutations, possibly sharing a common pathway—release of calcium from the ER into the cytosol via ITPR and RYR. UPR activation is an early event in the AD brain, which enhances autophagy but not the ubiquitin proteasome system in the phosphorylated EIF2AK3-positive neurons via increasing MAP1LC3B, suggesting that autophagy is the predominant pathway for degradation of Aβ or APP (Nijholt, de Graaf et al. 2011). Insufficient autophagic function is also suggested to play a significant role in the progression of AD (Pickford, Masliah et

al. 2008). The study by Pickford *et al.* demonstrated substantial loss of BECN1 in the midfrontal cortex of AD brains in the disease progression (Pickford, Masliah et al. 2008). Intriguingly, studies by this group also demonstrated that genetic reduction of BECN1 expression results in increased deposition of Aβ deposition and synaptic loss and, reciprocally, increasing BECN1 expression through administration of lentivirus alleviates amyloid pathology in the transgenic AD mouse model (Pickford, Masliah et al. 2008). In addition to neurons, loss of BECN1 in microglia, leading to impaired phagocytic clearance, is also a contributing factor for exacerbated Aβ accumulation (Lucin, O'Brien et al. 2013). The presence of Aβ also triggers inflammatory responses in microglia (Halle, Hornung et al. 2008). Phagocytosis of Aβ crystals by microglia disrupts the integrity of lysosome and the release lysosomal proteolytic enzyme CTSB (cathepsin B) activates NLRP3 (NLR family, pyrin domain containing 3) inflammasomes. This ultimately culminates in activation of the IL1B (interleukin 1, beta) pathway with subsequent generation of proinflammatory and neurotoxic factors (Halle, Hornung et al. 2008). Reciprocally, enhancing autophagic activity by genetic deletion of CSTB (cystatin B [stefin B]; an endogenous inhibitor of lysosomal cysteine proteases) or temsirolimus (a drug used for the treatment of renal cell carcinoma) reduces Aβ deposition and exerts protective effects in both cellular and mouse models of AD **(Figure 2.4)** (Yang, Stavrides et al. 2011, Jiang, Yu et al. 2014).
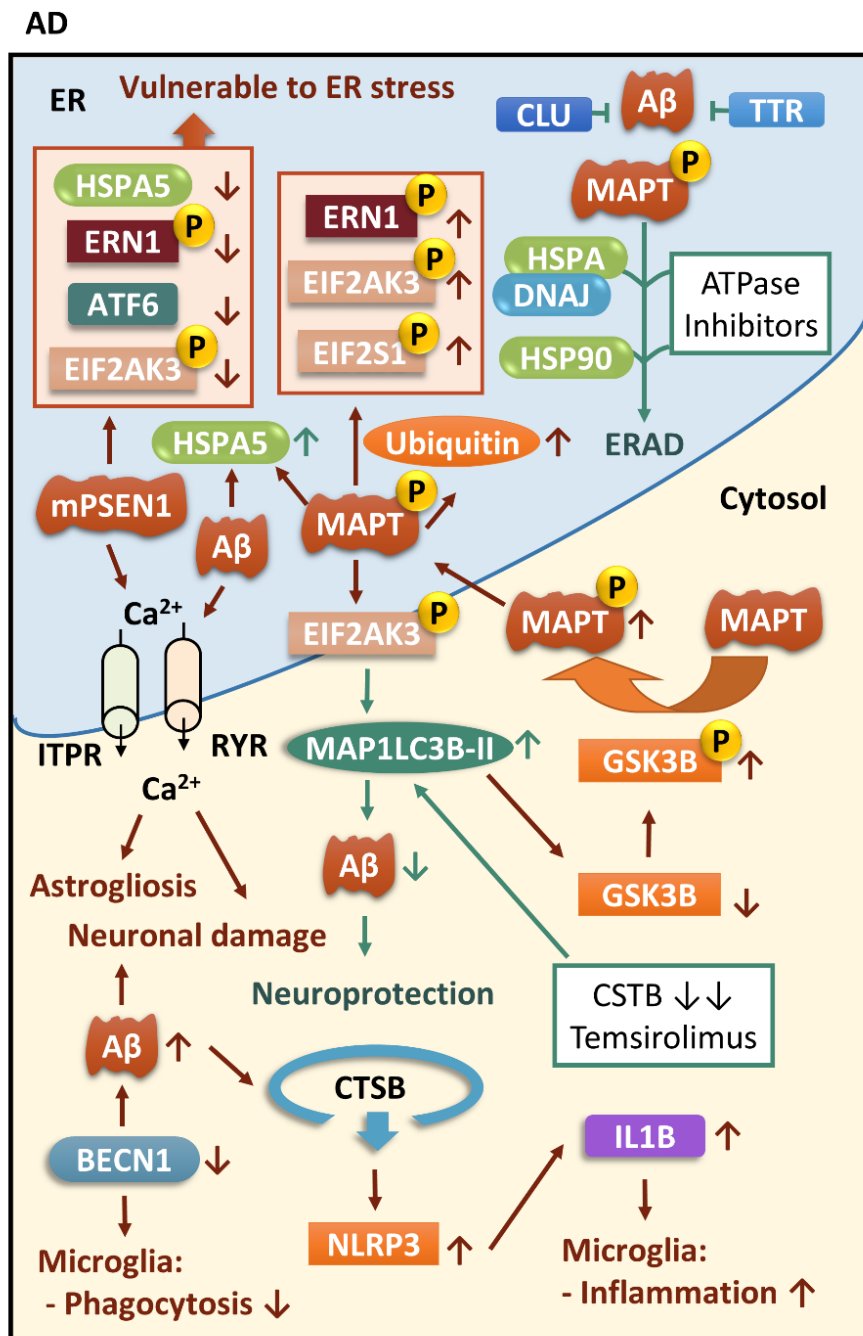
Figure 2.4 ER stress and autophagy in AD.

Both mPSEN1 (mutant PSEN1) and Aβ induce calcium-dependent ER stress, resulting in astrogliosis and neuronal injury. Loss of BECN1 not only contributes to neuronal damage but also debilitates the phagocytic function and induces inflammatory

responses in microglia. Interestingly, mPSEN1 renders neurons vulnerable to ER stress by suppressing major UPR mediators while MAPT protein enhances its phosphorylation through activation of UPR mediators, especially EIF2AK3. The EIF2AK3-dependent autophagy pathway is crucial for degradation of Aβ, and autophagic activities can be also enhanced by genetic deletion of CSTB/cystatin B or treatment with temsirolimus. However, autophagy mediated by EIF2AK3 signaling also activates GSK3B by removing its inactive form resulting in subsequent phosphorylation and aggregation of MAPT protein. Moreover, ATPase inhibitors for HSPA/DNAJ complexes and HSP90 multi-component complexes facilitate the triage of MAPT towards the ERAD pathway. CLU and TTR are able to bind to aggregated proteins to prevent their aggregation.

In summary, dysregulated UPR and impaired autophagy fail to eliminate pathogenic accumulation of abnormal proteins, resulting thereby in increased symptomatology of AD. Targeting these pathways could thus have therapeutic relevance for the treatment of AD.

In addition to Aβ accumulation, there is extensive evidence on the aggregation of yet another axonal cytosolic protein, the MAPT that is found hyperphosphorylated as in the brains of patients with AD, frontotemporal dementia (FTD), progressive supranuclear palsy (PSP), corticobasal degeneration, primary age-related tauopathy (PART) and traumatic brain injury (TBI) (Taylor, Hardy et al. 2002, Abisambra, Jinwal et al. 2013, Abisambra and Scheff 2014, Crary, Trojanowski et al. 2014). Mutations in the gene encoding MAPT are most commonly associated with tauopathies such as FTD, PSP and corticobasal degeneration, whereas other genetic risk factors such as mutations in C9orf72 (chromosome 9 open reading frame 72), GRN (granulin), the gene encoding VCP (valosin containing protein) and CHMP2B (charged multivesicular body protein 2B) affecting UPR and autophagy, play an essential role in frontotemporal lobar degeneration (FTLD) and AD (Sieben, Van Langenhove et al. 2012). Whereas the neurodegenerative pathology of tauopathies has been well recognized, the relevant molecular mechanism(s) underlying the disease processes remain poorly understood. Increasing evidence suggests that aggregated MAPT can lead to ER stress and activation of the UPR as an early event in tauopathies such as AD, FTD and PSP **(Figure 2.4)** (Hoozemans, van Haastert et al. 2012, Nijholt, van Haastert et al. 2012). In AD postmortem brain tissues, activation of UPR mediators such as EIF2AK3, ERN1 and HSPA5 is observed in the pretangled neurons containing diffusely distributed phosphorylated MAPT, but not in the neurons with densely aggregated neurofibrillary tangles (Hoozemans, Veerhuis et al. 2005, Hoozemans, van Haastert et al. 2009). Direct evidence of MAPT-dependent UPR is found in sporadic tauopathies lacking Aβ (Nijholt, van Haastert et al. 2012). For example, a report

by Nijholt *et al.* demonstrated that UPR activation is only observed in the MAPT subtype of FTLD, but not in other aggregate subtypes of FTLD (Nijholt, van Haastert et al. 2012). Elegant work by Abisambra *et al.* using MAPT transgenic mouse brains has also shown that MAPT increases the levels of ubiquitinated proteins in the brain and initiates the activation of the UPR as evidenced by increased levels of phosphorylated EIF2AK3. Findings from this group led to the suggestion that the presence of MAPT interferes with ER protein quality control (Abisambra, Jinwal et al. 2013). GSK3B (glycogen synthase kinase 3 beta) is an essential kinase for the phosphorylation of MAPT that plays an important role in MAPT aggregation (Nijholt, Nolle et al. 2013). Interestingly, the study by Nijholt *et al.* has shown that the EIF2AK3 pathway activates GSK3B through selective removal of inactive GSK3B via the autophagy-lysosomal pathway (Nijholt, Nolle et al. 2013). Moreover, findings by van der Harg *et al.* have also reported that failure to restore the metabolic homeostasis resulting in prolonged UPR activation and MAPT phosphorylation could likely be a contributing factor to AD pathogenesis. Furthermore, these authors also demonstrated the role of UPR-mediated MAPT phosphorylation as part of an adaptive response to metabolic stress, culminating ultimately into the pathogenesis of AD (van der Harg, Nolle et al. 2014).

Hyperphosphorylated MAPT is a target client that is recognized by the chaperone system and is processed initially by the HSPA/DNAJ (heat shock 70kDa proteins/DnaJ (Hsp40) homolog) complex followed by an intermediate complex with HSP90 (heat shock 90kDa protein) and STIP1 (stress-induced phosphoprotein 1) (Jinwal, Koren et al. 2010). Intriguingly, HSP90 regulates the folding or degradation of its client proteins by forming multi-component complexes with other chaperones. HSP90 has the ability to cycle between the different multi-chaperone complexes, from a complex favoring the refolding of abnormal proteins in the presence of ATP to a complex that targets proteins for

degradation (in the presence of ADP) (Hartl, Bracher et al. 2011). Inhibitors for heat shock proteins convert the HSP90 complexes from a catalyst for protein folding into one that promotes protein degradation (Jinwal, Koren et al. 2010). These inhibitors can thus be harnessed to convert the protein folding form to a protein degradation pathway, thereby allowing for the removal of abnormal phosphorylated MAPT. In keeping with this differential function of the chaperone system on toxicity, the extracellular chaperone CLU (clusterin) exhibits paradoxical roles in the pathology of AD depending on the concentration of misfolded proteins (Wilson, Yerbury et al. 2008). On the one hand, under normal conditions CLU can stabilize extracellular misfolded proteins via its binding to them and subsequently guiding them to specific cell surface receptors for uptake and degradation (Wu, Yu et al. 2012). Such extracellular chaperones and their receptors form a critical component of the quality control system to protect the host against abnormal, aggregated proteins such as Aβ (Wu, Yu et al. 2012). On the other hand, there is also evidence that in the presence of high levels of misfolded proteins, CLU can actually exacerbate the pathology (Wilson, Yerbury et al. 2008). Similar to CLU, TTR (transthyretin) can also regulate aggregated protein quality control by sequestering soluble Aβ and thereby preventing the formation of amyloid fibers (Sousa, Cardoso et al. 2007).

ER stress in AD is commonly observed in both Aβ and PSEN1 mutation and MAPT pathology. Interestingly, Aβ-induced calcium-dependent ER stress is suggested to be a conserved pathway that both neurons and astrocytes share in common (Suen, Lin et al. 2003, Alberdi, Wyssenbach et al. 2013). Loss of BECN1 impairs autophagy in AD brains and leads to synaptic injury in neurons, and phagocytic deficits in microglia, suggesting that autophagy is a conserved pathway and can affect the functioning of both neurons and glial cells (Pickford, Masliah et al. 2008, Lucin, O'Brien et al. 2013). Aβ-mediated microglial inflammation further suggests a crucial role of glial cells in modulating AD inflammatory

trajectory (Halle, Hornung et al. 2008). However, the UPR and autophagy play a protective role in neurotoxicity mediated by Aβ and PSEN1 mutations(Katayama, Imaizumi et al. 1999, Yu, Luo et al. 1999) but not in MAPT pathology (Nijholt, Nolle et al. 2013). The phosphorylated EIF2AK3-dependent autophagy pathway is considered as the major degradation pathway for Aβ (Nijholt, de Graaf et al. 2011), but is also capable of activating GSK3B by degrading its inactive forms, leading to MAPT phosphorylation and aggregation (Nijholt, Nolle et al. 2013). Thus, intervention in the UPR and autophagy in AD can be considered as potential therapeutic targets; however, the genetic profiling and pathology of AD patients should be carefully investigated when designing potential therapeutic strategies based on the perspective of the UPR and autophagy.

2.6 Prion diseases

Prion diseases, also known as transmissible fatal neurodegenerative diseases, are characterized by neuronal loss, synaptodendritic degeneration, spongiosis, gliosis and deposition of insoluble PRNP (prion protein) aggregates in the brain (Mead 2006, Hetz, Castilla et al. 2007, Dearmond and Bajsarowicz 2010). PRNP exists as a native form of prion protein PRNP^C in the non-infected brain, and as a protease-resistant form of prion protein PRNP^{SC} in the prion diseased brain (Hetz and Soto 2006). In prion diseases, PRNP^C is converted into PRNP^{SC} that is rich in β-sheet and prone to intracellular accumulation, resulting in ER stress (Hetz and Soto 2006). The conversion also depletes PRNP^C in neurons and stimulates synthesis of PRNP^C for further cycles of conversion, thereby exacerbating ER stress (Hetz, Castilla et al. 2007). A similar template for conformational change is also observed in other prion-like disease-specific proteins

including MAPT, Aβ and SNCA (Petkova, Leapman et al. 2005, Desplats, Lee et al. 2009, Frost, Jacks et al. 2009).

Accumulating evidence suggests active ER stress responses in prion diseases both *in vitro* and *in vivo* **(Figure 2.5)** *(Hetz, Russelakis-Carneiro et al. 2003, Hetz, Russelakis-Carneiro et al. 2005, Ferreiro, Costa et al. 2008, Wang, Shi et al. 2012)*.
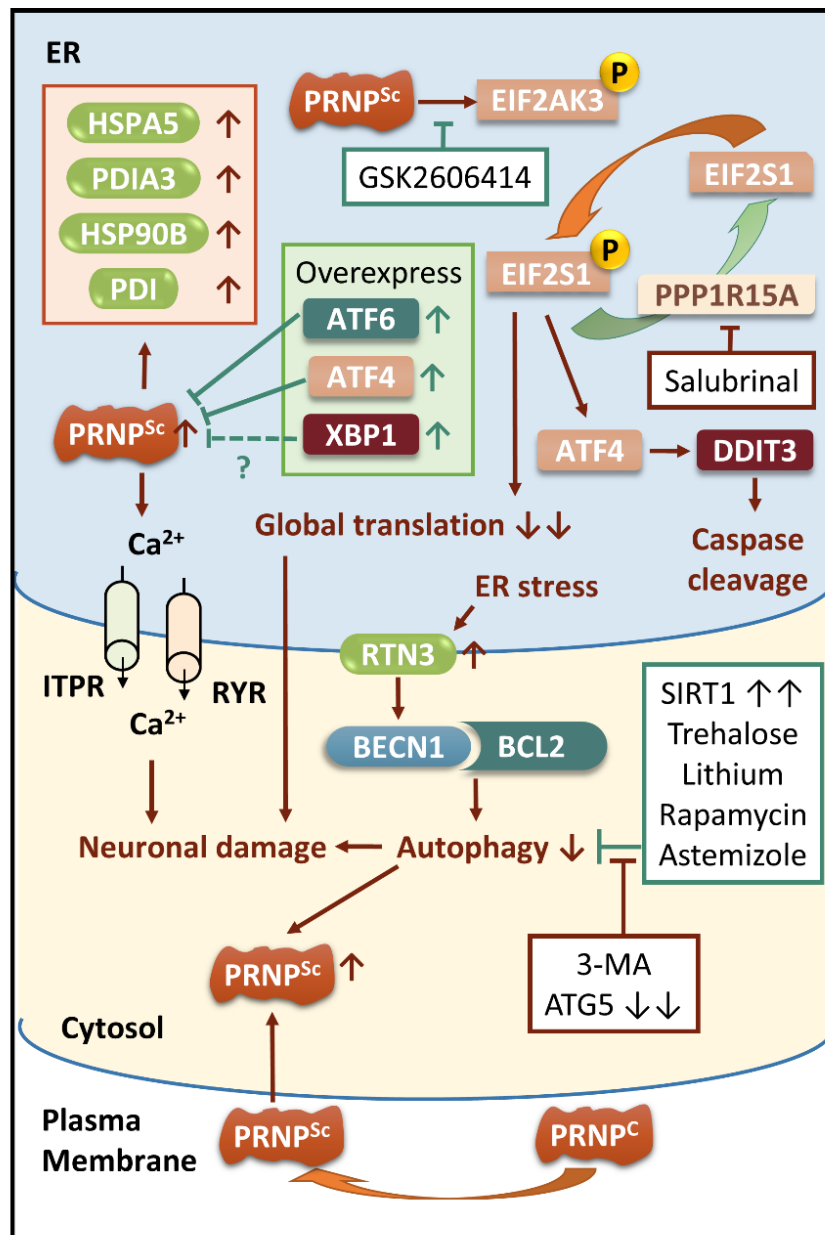
Figure 2.5 ER stress and autophagy in prion diseases.

ER stress induced by disruption of intracellular calcium homeostasis is also observed in prion diseases. Aggregation of PRNP upregulates ER chaperones and activates EIF2AK3-EIF2S1 signaling, resulting in synaptic failure and activation of a

caspase cascade, which can be rescued by the EIF2AK3 inhibitor GSK2606414 but not the PPP1R15A inhibitor salubrinal. Overexpression of UPR mediators such as ATF6, ATF4 and XBP1 reduces PRNP aggregation *in vitro,* but it is not fully extrapolated in XBP1 conditional knockout mice. RTN3 activated by ER stress restrains autophagy by prohibiting dissociation of BCL2 and BECN1. Autophagic activities can be enhanced by overexpression of SIRT1 and autophagic inducers, and can be inhibited by either genetic silencing of ATG5 or in the presence of the autophagic inhibitor 3-MA.

Transcriptional analysis of brainstem tissues from 100 confirmed cases of prion diseases in cattle found increased expression of ER chaperones, ERAD components, regulators of ER calcium storage and mediators of ER stress-induced apoptosis (Tang, Xiang et al. 2010). Upregulation of ER chaperones HSPA5, PDIA3 (protein disulfide isomerase family A, member 3), and HSP90B1 (heat shock protein 90kDa beta [Grp94], member 1) has been reported in N2a neuroblastoma cells infected with scrapie *in vitro* and scrapie rodents models *in vivo* (Hetz, Russelakis-Carneiro et al. 2003, Hetz, Russelakis-Carneiro et al. 2005, Ferreiro, Costa et al. 2008, Wang, Shi et al. 2012). Prion proteins also increase the release of ER calcium into cytosol, induce inhibition of global protein synthesis and cause dysregulation of autophagy. PRNP can induce ER stress-dependent apoptosis through disruption of calcium homeostasis via ITPR and RYR in primary neurons (Ferreiro, Resende et al. 2006). Although overexpression of UPR mediators decreases PRNP aggregation in cell lines *in vitro* (Orsi, Fioriti et al. 2006, Hetz, Castilla et al. 2007), the same phenomenon cannot be fully extrapolated in neurons in XBP1 conditional knockout mice (Hetz, Lee et al. 2008, Torres, Castillo et al. 2010). Studies by Mallucci and Moreno demonstrated that in response to accumulation of PRNP, global protein synthesis in prion-diseased mice is inhibited by phosphorylation of EIF2S1 leading to synaptic failure and neuronal loss (Moreno, Radford et al. 2012). This group further demonstrated that targeting the upstream ER stress molecule EIF2S1 is able to reverse cognitive deficits and prevent clinical signs of prion disease at the preclinical stage (Moreno, Halliday et al. 2013). Intriguingly, the ER-resident protein RTN3 (reticulon 3) that can be activated under ER stress is reported to play an important role in the cytosolic PRNP[SC] aggregation in N2a cells infected with the prion protein fragment PRNP (90-231). RTN3 restrains autophagic activity by enhancing interaction between BCL2 and BECN1, and knockdown of RTN3 not only promotes the autophagic clearance of PRNP[SC], but alleviates the ER stress and improves cellular survival (Chen, Jin et al. 2011).

It has been suggested that insufficient cellular clearance in prion diseases can be partially related to dysregulation of autophagy **(Figure 2.5)** (Rubinsztein, DiFiglia et al. 2005). Intracellular autophagic vacuoles/autophagosomes of different sizes are found located in neuronal perikarya, neurites and synapses in various prion-like diseases (Sikorska, Liberski et al. 2004). It has been reported that adenoviral-mediated SIRT1 (sirtuin 1) overexpression enhances autophagic activity to protect human neuroblastoma SH-SY5Y cells from neurotoxicity, mitochondrial injury and an intrinsic apoptosis pathway that are meditated by the prion peptide PRNP(106-126). Knocking down ATG5 abrogates this effect (Jeong, Moon et al. 2013). The autophagic inducers trehalose and lithium diminish accumulation of PRNP^{SC} in ScN2a cells (N2a cells persistently infected with prion strain RML) and this protective effect can be blocked either with 3-methyladenine (3-MA; an inhibitor of phosphatidylinositol 3-kinase that inhibits cellular autophagy) or by genetically silencing ATG5 (Aguib, Heiseke et al. 2009, Heiseke, Aguib et al. 2009). Intraperitoneal injection of the classic autophagic inducer rapamycin in a transgenic (PRNP-A116V) Gerstmann-Sträussler-Scheinker mouse model was also demonstrated to decrease PRNP^{SC} accumulation, induce a dose-dependent delay in disease onset, alleviate the disease symptoms and extended the life span (Cortes, Qin et al. 2012). Using a PRNP-FRET-enabled high-throughput assay to screen for compounds that decrease PRNP expression, a recent study on CJD demonstrated that another autophagic inducer, astemizole (originally used to treat chronic seasonal allergic rhinitis), prolongs the survival time of prion-infected mice, suggesting thereby the possibility of therapeutic use of astemizole in CJD (Karapetyan, Sferrazza et al. 2013).

Taken together, prion protein induces the release of ER calcium into cytosol, induces inhibition of global protein synthesis and causes dysregulation of autophagy. Therefore, maintaining the intracellular calcium homeostasis, counteracting the global

translation repression and enhancing autophagic activities can be considered as promising therapeutic strategies for combating prion diseases.

2.7 Parkinson's disease

PD is the second most common neurodegenerative disorder afflicting the elderly and is usually characterized by hypokinesia, rigidity and tremor. The extensive degeneration of dopaminergic neurons in the *substantia nigra pars compacta* accounts for the majority of the symptomatology (Lang and Lozano 1998, Lang and Lozano 1998). The salient pathological features of PD includes accumulation of Lewy bodies, intracellular inclusion bodies containing diffuse deposits of misfolded SNCA protein (Pollanen, Dickson et al. 1993). Widely accepted PD models mimic apoptosis of dopaminergic neurons through administration of PD-related insults such as 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-pyridinium (MPP$^+$), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone (Ryu, Harding et al. 2002, Sado, Yamasaki et al. 2009).

In PD, phosphorylated forms of the UPR activation marker EIF2AK3 colocalize with the SNCA protein in the human substantia nigra, indicating thereby induction of ER stress and its association with the aggregated SNCA (Hoozemans, van Haastert et al. 2007). Similar findings have been demonstrated in chemically-induced experimental models of PD. Following exposure to 6-OHDA, there is increased phosphorylation of EIF2AK3 and ERN1 in neurons, furthermore, it sympathetic neurons from EIF2AK3 null mice exhibit extended sensitivity to 6-OHDA (Ryu, Harding et al. 2002). In addition to phosphorylated EIF2AK3, exogenous XBP1 protein also protects dopaminergic neuroblastoma SK-N-SH cells from MPP$^+$ and proteasome inhibitors, and additionally, adenoviral *XBP1s* suppresses MPTP-mediated degeneration of dopaminergic neurons in

mouse models (Sado, Yamasaki et al. 2009). The role of EIF2S1 in the phenotype of α-synucleinopathy, however, remains unclear. Although phosphorylated EIF2S1 is present in the substantia nigra of PD individuals (Hoozemans, van Haastert et al. 2007), it is not found to be activated in the α-synucleinopathy mouse model (*A53TαS* Tg) (Colla, Coune et al. 2012). Interestingly, DDIT3, a pro-apoptotic mediator in the final stage of ER stress, is a mediator of neuronal cell death in the rat substantia nigra induced by 6-OHDA but not MPTP. Upregulation of DDIT3 is observed in dopaminergic neuronal cell death mediated by both 6-OHDA and MPTP; however, a null mutation of DDIT3 *in vivo* protects mice from only 6-OHDA-induced apoptosis **(Figure 2.6)** (Silva, Ries et al. 2005).
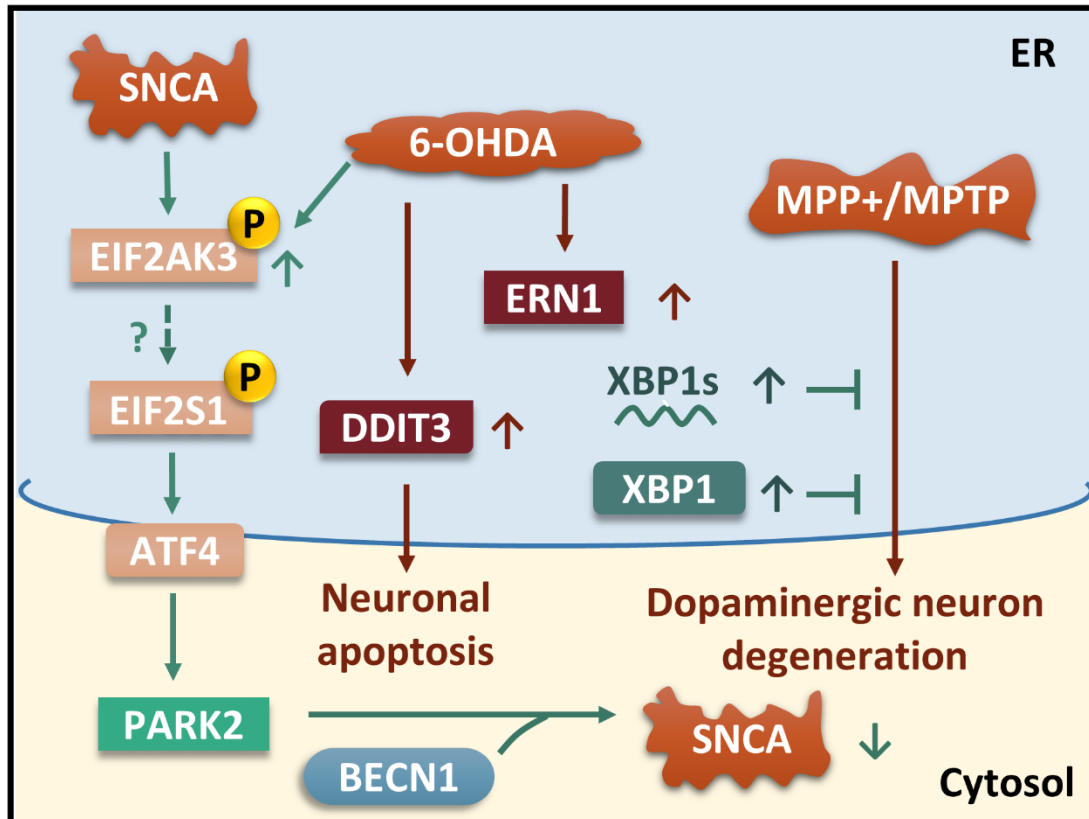
Figure 2.6 ER stress and autophagy in PD.

Phosphorylation of EIF2AK3 can be induced by both SNCA and 6-OHDA. Although phosphorylated EIF2S1 is seen in PD brain but not in the α-synucleinopathy mouse model, EIF2S1-ATF4 signaling upregulates PARK2. Interaction of PARK2 and BECN1 enhances autophagic clearance of SNCA. Moreover, exogenous *XBP1s* and XBP1 rescue MPP[+]/MPTP-mediated dopaminergic neuron degeneration.

Accumulating evidence thus suggests that UPR regulators such as EIF2AK3 and *XBP1s* play important roles in neuroprotection in PD, however, more evidence is needed to clarify the roles of EIF2S1 and ERN1 in this process. Although DDIT3 is usually associated with ER stress-associated apoptosis, it appears that the nature of the toxic stimulus determines whether DDIT3 will induce neuronal apoptosis in PD.

Chaperone-mediated autophagy is crucial for the clearance of soluble SNCA protein in cultured dopaminergic neurons because the SNCA protein contains a pentapeptide sequence ($_{95}$VKKDQ$_{99}$), and its degradation can be blocked by the lysosomal proteolysis inhibitor ammonium chloride (Cuervo, Stefanis et al. 2004). PARK2 (parkin RBR E3 ubiquitin protein ligase) is a ubiquitin ligase reported to localize in mitochondria (Kuroda, Mitsui et al. 2006), mediate the engulfment of mitochondria by phagophores, selectively eliminate dysfunctional mitochondria (Ashrafi and Schwarz 2013) and rescue the inhibition of mitochondrial fusion induced by SNCA (Kamp, Exner et al. 2010). Dopaminergic neuroblastoma-derived SH-SY5Y cells transfected with PARK2 are more resistant to cell death induced by tunicamycin (*N*-glycosylation inhibitor)-mediated ER stress (Imai, Soda et al. 2000). Silencing EIF2S1 and its downstream target ATF4 reduces PARK2 expression in mouse primary cortical neurons and disrupts mitophagic function, leading to increasing neuronal cell death (Zhang, Yuan et al. 2014). Interestingly, although PARK2 colocalizes with BECN1 in human mid-brain, it has decreased interaction with BECN1 in sporadic PD cases. Furthermore, there was increased secretion of SNCA from the brain into the blood in PARK2-deficient mice **(Figure 2.6)** (Lonskaya, Desforges et al. 2013).

Different PD insults all trigger ER stress and activate the UPR that is suggested to play a neuroprotective role (Ryu, Harding et al. 2002, Sado, Yamasaki et al. 2009). PARK2 is a critical neuroprotective molecule bridging the UPR and autophagy in PD and thus can

be considered as a promising target for therapeutic purposes (Zhang, Yuan et al. 2014). Although upregulation of DDIT3 is a common feature in different PD models, DDIT3 mediates neuronal apoptosis in the presence of 6-OHDA but not in the presence of MPTP (Silva, Ries et al. 2005), an effect that is dependent on the nature of the PD stimulants. Due to the fact that phosphorylated EIF2S1 is present in human PD brains but not in the *A53TαS* transgenic mouse model, more evidence is needed to clarify the roles of EIF2S1 in this process (Hoozemans, van Haastert et al. 2007, Colla, Coune et al. 2012).

2.8 Amyotrophic lateral sclerosis

ALS, often referred to as "Lou Gehrig's disease", is a progressive and fatal adult-onset neurodegenerative disorder with a selective degeneration and loss of motor neurons, characterized by muscle weakness, atrophy, spasticity, paralysis and premature death (Pasinelli and Brown 2006). Whereas most of the ALS cases are sporadic ALS (sALS), nearly 10% of ALS cases are familial ALS (fALS) (Gurney, Pu et al. 1994, Pasinelli and Brown 2006). It has been reported that abnormal aggregation of mutant (m)SOD1 (superoxide dismutase 1, soluble) accounts for about 20 % of fALS cases(Strong, Kesavapany et al. 2005, Atkin, Farg et al. 2008) and 1-7 % of sALS cases. Additionally, mutations in genes, such as those encoding TARDBP (TAR DNA binding protein), FUS/TLS (FUS RNA binding protein), and OPTN (optineurin) also are reported to be a causative factor of ALS (Christensen, Fallingborg et al. 1990, Kabashi, Valdmanis et al. 2008, Del Bo, Ghezzi et al. 2009).

Various reports suggest that aggregation of mSOD1 results in prolonged ER stress leading to the loss of motor neurons through the activation of pro-apoptotic factors such

as CASP3, ATF3, DDIT3 and BH3-only proteins, in the late stages of SOD1^{G93A} ALS mouse models (Vlug, Teuling et al. 2005, Atkin, Farg et al. 2006, Kieran, Woods et al. 2007). An increase in HSPA5,[193] PDI (Atkin, Farg et al. 2008), EIF2S1 (Ilieva, Ayala et al. 2007), ATF4 and *XBP1s* (Hetz, Thielen et al. 2009) was also reported in the post-mortem brains of ALS patients. The chronic ER stress in fALS patients is likely to be associated with the suppression of compensatory autophagic clearance of mSOD1. One of the ER-stress regulators, XBP1, suppresses autophagic clearance of mSOD1 possibly via inhibition of MTORC1 **(Figure 2.7)** (Hetz, Thielen et al. 2009).
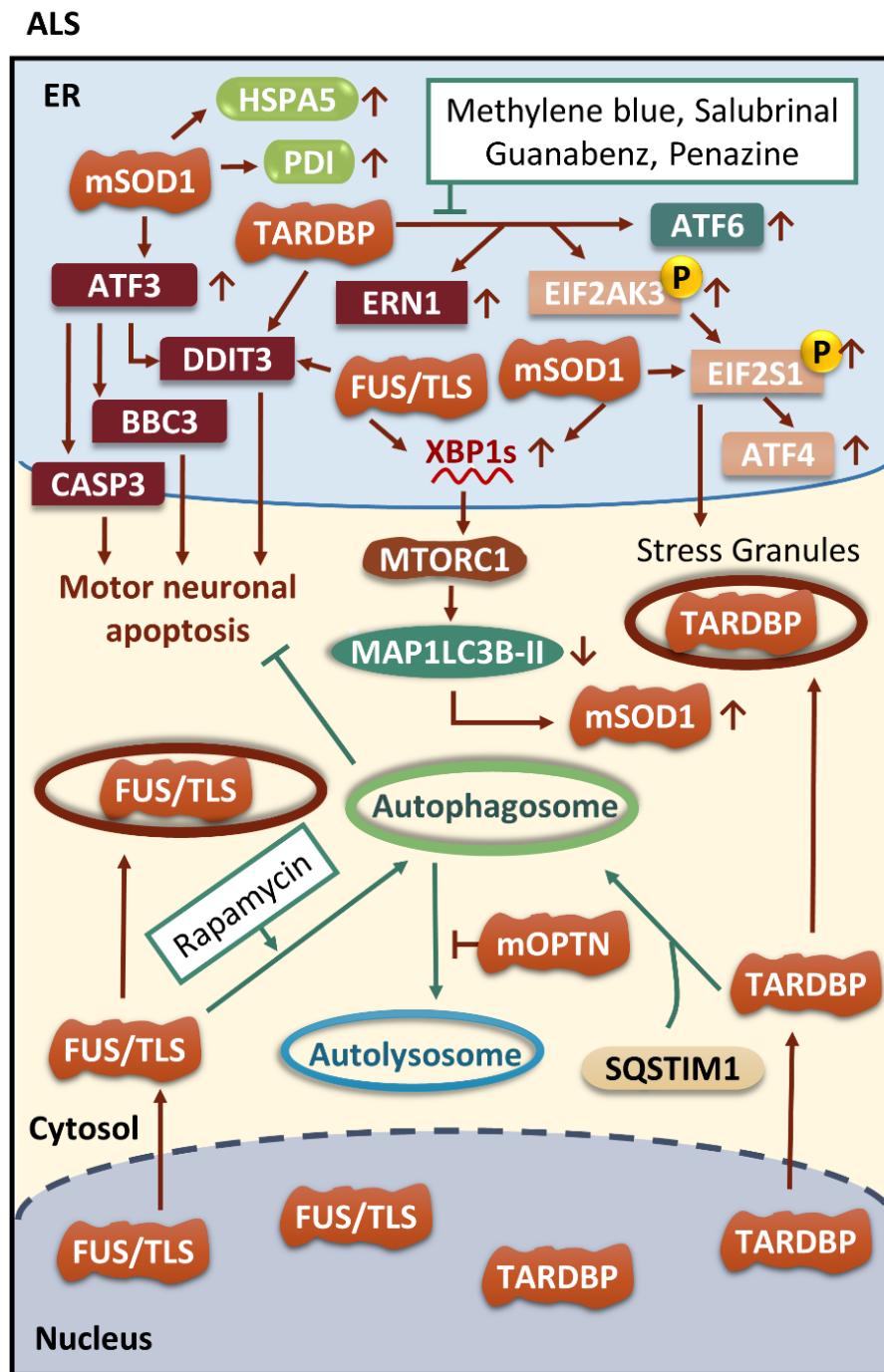
Figure 2.7 ER stress and autophagy in ALS.

Different mutations such as those in SOD1, TARDBP and FUS are all able to induce neuronal apoptosis through activation of DDIT3. Both TARDBP and FUS can be

included in stress granules in the cytoplasm. Phosphorylation of EIF2S1 mediated by TARDBP and mSOD1 (mutant SOD1) facilitates stress granules formation. Interestingly, either FUS or mSOD1 increases expression of *XBP1s* but activated *XBP1s* inhibits autophagic clearance of mSOD1. Moreover, mOPTN (mutant OPTN) also hampers autophagy by inhibiting fusion of autophagosomes with lysosomes. Enhanced autophagic activity induced by autophagic inducers leads to clearance of FUS and TARDBP. Interestingly, SQSTM1 plays a crucial role in packaging TARDBP into autophagosomes.

Targeting inhibition of XBP1 and enhancing autophagic clearance can be considered a promising therapeutic strategy for clearing mSOD1 in ALS.

TARDBP, a nuclear RNA-binding protein that plays a critical role in RNA processing as well as microRNA biogenesis modulation, continually shuttles between the nucleus and the cytoplasm (Ayala, Zago et al. 2008, Kawahara and Mieda-Sato 2012). Under normal conditions, TARDBP is processed and degraded by both autophagy and the ubiquitin-proteasome systems. When TARDBP is excluded from the nucleus it forms cytoplasmic aggregates in the brain and spinal cord of ALS patients (Barmada, Skibinski et al. 2010). Over 30 mutations have been reported in the TARDBP gene to cause fALS; such mutations endorse the cytoplasmic mislocalization and accumulation of TARDBP leading to cytoplasmic TARDBP toxicity (Barmada, Skibinski et al. 2010). The aggregated TARDBP also triggers ER stress both *in vitro* and *in vivo* (Farg, Soo et al. 2012, Walker, Soo et al. 2013). The aggregated TARDBP can also be phosphorylated, ubiquitinated and degraded by the ubiquitin-proteasome system and autophagy via degradation target proteins such as UBQLN1 (ubiquilin 1) and UBQLN2 (ubiquilin 2), and SQSTM1 (sequestosome 1), respectively **(Figure 7)** (Bose, Huang et al. 2011, Majcher, Goode et al. 2015). Indeed, various studies demonstrated a failure of TARDBP clearance in human ALS. Additionally, *in vitro* studies demonstrated that the primary mutation of TARDBP confers resistance to its degradation (Ling, Albuquerque et al. 2010).

In addition to TARDBP, another RNA metabolism-associated protein, FUS, has been identified as a key constituent within the intracellular protein inclusions in the autopsy tissues of ALS (Ling, Albuquerque et al. 2010). FUS is predominantly restricted in the nucleus with little distribution in the cytoplasm of motor neurons; however, point mutations in the C terminus of FUS cause neuronal cytoplasmic FUS-positive inclusions in ALS patients (Dormann, Rodde et al. 2010). Hence, FUS reshuffles from the nucleus to the

cytoplasm and builds inclusions in affected motor neurons, where it initiates ER stress in motor neuron-like cells expressing mutant FUS. The ER chaperone PDI was observed to colocalize with the mutant FUS in motor-neuron-like NSC-34 cells as well as in the FUS inclusions in human ALS lumbar spinal cords, in both sALS and mutant FUS-linked fALS tissues (Farg, Soo et al. 2012). It was also reported that the point mutation in FUS diminishes the release of FUS from accumulating stress granules in cultured neurons leading to defective autophagy clearance, thereby indicating that activation of autophagic signaling could be a therapeutic approach for FUS mutation-associated ALS pathologies **(Figure 2.7)** (Ryu, Jun et al. 2014).

OPTN is an autophagy receptor protein, and mutations in OPTN have recently been reported as a causative factor for ALS and glaucoma (Maruyama and Kawakami 2013). OPTN is primarily involved in autophagosome maturation by fusion with endosomes and its MYO6/myosin VI binding partner (Tumbarello, Waxse et al. 2012). OPTN is also considered as a neuroprotective protein, whose accumulation is normally cleared by the proteasome degradation pathway. The OPTN E478G mutation, a causative mutation for ALS, is reported to effectively impair autophagosome recruitment, which further confirms the involvement of OPTN mutation and defective autophagy in the pathology of ALS **(Figure 2.7)** (Wong and Holzbaur 2014). Thus, reducing ER stress and maintaining active autophagy can be considered as promising therapeutic targets for ALS.

2.9 HIV associated-neurocognitive disorders

In the era of combined anti-retrovirus therapy, the prevalence of HAND is actually on a rise due to a significant increase in the longevity of the infected patients (Fields,

Dumaop et al. 2013, Yang, Yao et al. 2015). Mechanisms underlying HAND pathogenesis, however, remain unclear. Recent emerging studies provide ample evidence indicating that early HIV proteins that are not affected by combined anti-retroviral therapy and that can deregulate the ER stress pathways and autophagy activity play a crucial role in the development of HAND.

Tat (Transactivator of transcription), one of the key HIV proteins that is necessary for virus replication, is capable of activating ER stress pathways in various types of cells in the brain (Tumbarello, Waxse et al. 2012, Wong and Holzbaur 2014). Studies from our lab have demonstrated that Tat-induced apoptosis of human brain microvascular endothelial cells involves ER stress activation and mitochondrial dysfunction (Ma, Yang et al. 2014). Tat also upregulates ER stress markers including HSPA5, ATF6 and phosphorylated EIF2S1 in both cortical neurons and astrocytes (Akay, Lindl et al. 2012). The fluctuation of cytosolic Ca^{2+} concentration is the upstream signal for ER activation as evidenced by the depletion of Ca^{2+} from the ER to the cytoplasm via ITPR and RYR (Haughey, Holden et al. 1999, Norman, Perry et al. 2008). Besides Tat, the virus envelope protein Gp120 also modulate ER stress in neurons, which in turn, has been suggested to correlate with neuronal injury (Caporello, Nath et al. 2006). Activation of ER stress contributes to increased permeability of the blood-brain barrier, and the activation of microglia and astrocytes, thereby resulting in enhanced expression of pro-inflammatory factors, and impairment of brain glutamate homeostasis, leading ultimately to exacerbated brain inflammation, a hallmark feature of HAND pathogenesis (Akay, Lindl et al. 2012, Ma, Yang et al. 2014, Guo, Liao et al. 2015). In addition to regulating ER stress activity, HIV proteins also modulate autophagy levels as demonstrated by autophagy dysfunction in HIV brains (Alirezaei, Kiosses et al. 2008, Alirezaei, Kiosses et al. 2008, Zhou, Masliah et al. 2011, Fields, Dumaop et al. 2013). HIV Gp120 and Nef have the ability to affect

autophagy levels by targeting various steps of the autophagy pathway (Kyei, Dinkins et al. 2009, Fields, Dumaop et al. 2013). For example BECN1 levels are altered in HIV Gp120 transgenic mice(Ryu, Jun et al. 2014), whereas Nef blocks the autophagosome maturation step via interaction with BECN1 (Kyei, Dinkins et al. 2009). Through the interaction of Nef and BECN1, HIV can decrease autophagic influx in infected cells thereby allowing for its replication (Kyei, Dinkins et al. 2009). HIV Tat also plays a key role in autophagy during the process of HIV infection. Tat suppresses autophagy in macrophages (Li, Au et al. 2011) and bystander monocytes (Van Grol, Subauste et al. 2010). Noticeably, Tat can also alter neuronal autophagy levels by blocking autophagosome fusion with the lysosome by disrupting lysosome membrane integrity, which is critical for the maintenance of normal lysosomal functioning **(Figure 2.8)** (Hui, Chen et al. 2012, Fields, Dumaop et al. 2015).
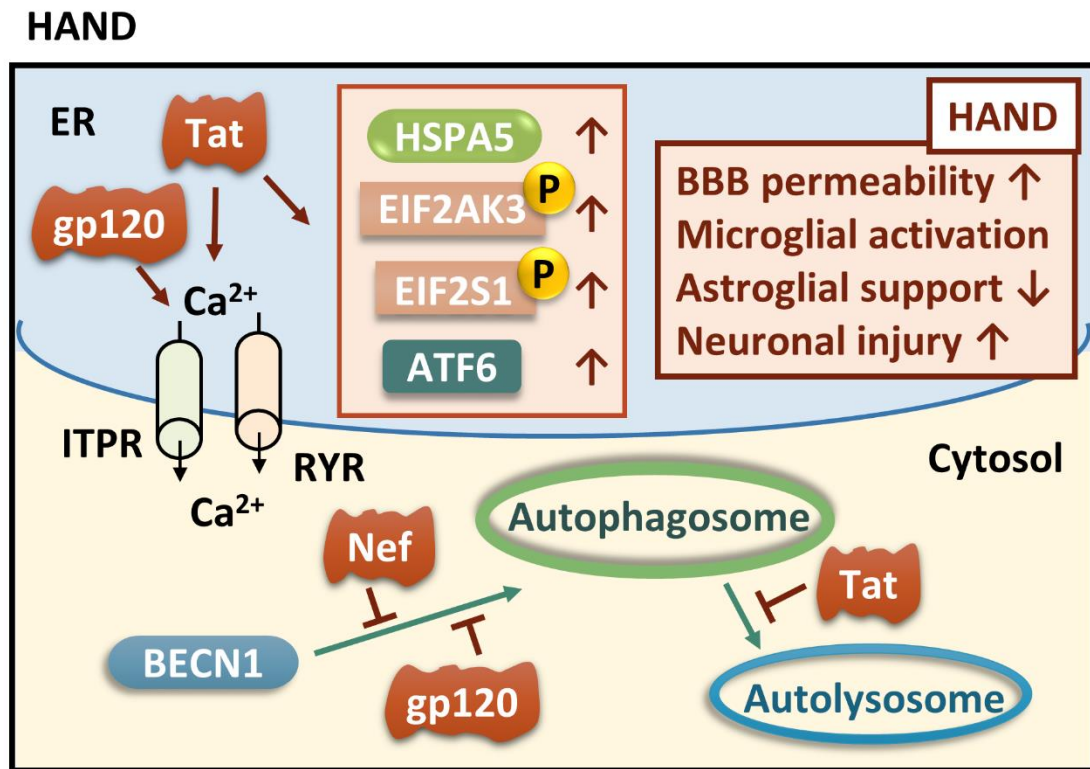
Figure 2.8 ER stress and autophagy in HAND.

Although no direct evidence is found between UPR and autophagy in HAND, it has been suggested that HIV viral proteins induce ER stress and inhibit autophagic clearance. Both Gp120 and Tat induce calcium-dependent ER stress, and Tat increases expression of major UPR mediators such as HSPA5, ATF6, phosphorylated EIF2AK3 and EIF2S1. Viral proteins such as Nef and Gp120 hamper autophagosome formation initiated by BECN1 while Tat hinders the fusion of lysosomes with autophagosomes. BBB, blood-brain barrier.

Autophagy dysfunction in HIV patients has also been linked with aging. Intriguingly, young (<50 y) versus aging (> 50 y) HIV patients reveal opposite trends in autophagic activity, with increased autophagy observed in younger compared to older HIV-infected individuals. This phenomenon suggests that different autophagy-targeting therapeutic strategies will be needed for young versus the old HIV-infected patients to achieve a beneficial clinical outcome.

Taken together, HIV proteins can affect both ER stress and autophagy activity to contribute to the development of HAND. In most cases, ER stress is the upstream signal for autophagy induction, and autophagy in turn can modulate the tone of ER stress via a feedback mechanism. In HAND, it is still not clear whether HIV proteins can sequentially first upregulate the levels of ER stress followed by induction of autophagy or if they cause the induction of both ER stress and autophagy simultaneously, or whether autophagy upregulation precedes ER stress. Additionally, unlike other neurodegeneration diseases, there is no clear evidence as to whether targeting the autophagy pathway can be considered an effective alternative therapeutic approach to ameliorate the symptoms of HAND. Further investigation is warranted in this area.

2.10   Conclusions and future directions

ER stress in neurodegenerative disorders can be triggered by either pathogenic accumulation of misfolded proteins such as Aβ in AD (Selkoe 2001, Chen, Gamache et al. 2014), PRNP^{SC} in prion diseases (Hetz and Soto 2006), SNCA in PD (Crary, Trojanowski et al. 2014) and mSOD1 in fALS (Atkin, Farg et al. 2006), or depletion of ER calcium stores via the ITPR and RYR channels in HAND (Haughey, Holden et al. 1999, Norman, Perry

et al. 2008) and AD (Suen, Lin et al. 2003). Intriguingly, Aβ-induced calcium-dependent ER stress is also suggested to be a conserved pathway that both neurons and astrocytes share in common that leads to neuronal damage and disruption of trophic support from astrocytes (Suen, Lin et al. 2003, Alberdi, Wyssenbach et al. 2013). Pathogenic accumulation of misfolded proteins leading to activation of the UPR, results in upregulation of mediators such as HSPA5, and the phosphorylated forms of EIF2AK3 and EIF2S1, which have been widely used as indicators of ER stress in various neurodegenerative disorders (Hoozemans, van Haastert et al. 2007, Ilieva, Ayala et al. 2007, Norman, Perry et al. 2008, Sasaki 2010). Generic cells such as HEK293 are powerful tools for studying ER stress. Using a novel methodology that activates the transcription factors XBP1 and/or ATF6 orthogonally by small molecules in the same cell, Shoulders *et al.* were able to demonstrate remodeling of the ER proteostasis network via the UPR transcriptional programs at the molecular level independent of stress (Shoulders, Ryno et al. 2013). Although most of the experiments in non-neuronal cells can be extrapolatable to neurons *in vivo*, exceptions do exist and therefore rodent models and human brain tissues are indispensable for investigating neurodegenerative disorders. For instance, overexpression of UPR mediators such as ATF6, ATF4 and XBP1 decrease PRNP aggregation in cell lines *in vitro* (Orsi, Fioriti et al. 2006, Hetz, Castilla et al. 2007), but the same phenomenon cannot be fully extrapolated to neurons in XBP1 conditional knockout mice (Hetz, Lee et al. 2008, Torres, Castillo et al. 2010). Under most circumstances, upregulated expression of UPR mediators is a pathway for protecting neurons against ER stress; however, it must be cautioned that not all upregulated UPR mediators confer neuroprotection. For example, in fALS upregulation of *XBP1* mRNA decreases MAP1LC3B-II, thereby inhibiting autophagic clearance of mSOD1 (Hetz, Thielen et al. 2009). In prion diseases, the EIF2S1-dependent prolonged global suppression of protein translation leads to synaptic failure and neuronal loss (Van Deerlin, Leverenz et al. 2008,

Gitcho, Strider et al. 2009, Deng, Chen et al. 2011, Dickson, Kouri et al. 2011, Hortobagyi, Troakes et al. 2011, Moreno, Radford et al. 2012, Simon-Sanchez, Dopper et al. 2012). In tauopathies, activation of GSK3B by the EIF2AK3 pathway results in phosphorylation of MAPT in the lysosome (Nijholt, de Graaf et al. 2011, Nijholt, Nolle et al. 2013). These UPR mediators can thus be envisioned as potential therapeutic targets. Depending on the nature of the toxic stimuli and the signaling pathway, both upregulation and downregulation of UPR mediators can direct the course of disease pathogenesis. For instance, in AD, Aβ induces expression of the ER chaperone HSPA5 to rescue abnormal accumulation of Aβ, whereas a neuronal cell line stably expressing mutant PSEN1 is more vulnerable to ER stress stimuli since mutant PSEN1 inhibits the activity of ER stress sensors HSPA5, ERN1 and ATF6 (Katayama, Imaizumi et al. 2001, Kudo, Katayama et al. 2002). Furthermore, maintaining a constant level of ER calcium is essential for the normal functioning of ER-resident molecular chaperones. There is also emerging evidence that pharmacological inhibitors for ITPR and RYR channels can rescue ER-stress associated neuronal apoptosis in HAND (Haughey, Holden et al. 1999) and AD (Suen, Lin et al. 2003), indicating thereby an important role for loss of the ER calcium store in the neurodegenerative processes.

Emerging evidence suggests that autophagy serves as an intrinsic compensatory mechanism for the UPR to regulate protein homeostasis and clear cell debris and misfolded proteins in neurodegenerative disorders. In the brains of AD patients, increasing MAP1LC3B levels have been observed in the phosphorylated-EIF2AK3-positive neurons (Nijholt, de Graaf et al. 2011). The ubiquitin ligase PARK2 plays a vital role in the interplay between ER stress and autophagy. Decreased interaction between PARK2 and BECN1 is involved in increased secretion of SNCA from the brain into the blood in the mouse model of PD (Lonskaya, Desforges et al. 2013). Furthermore, dopaminergic

neuroblastoma-derived SH-SY5Y cells transfected with PARK2 are more resistant to apoptosis mediated by ER stress (Imai, Soda et al. 2000). Some pathogenic proteins such as HIV Tat can escape autophagic clearance via interference with the endolysosomal pH, inactivation of endolysosomal enzymes and inhibition of autophagy (Hui, Chen et al. 2012). Interestingly, UPR mediators such as *XBP1s* can also hamper autophagic activity by decreasing the expression levels of MAP1LC3B-II in fALS (Hetz, Thielen et al. 2009). It is possible that neurons and non-neuronal cells such as microglia share a common pathway in autophagy. For instance, loss of the autophagy initiator BECN1 increases intracellular deposition of Aβ in neurons (Pickford, Masliah et al. 2008) and decreases phagocytic clearing, which exacerbates Aβ in microglial cells (Lucin, O'Brien et al. 2013). Moreover, the dysregulation of autophagy also contributes to neuroinflammation mediated by glial cells. With the loss of BECN1, the capacity of handling Aβ is diminished in microglia (Lucin, O'Brien et al. 2013) and aggregated Aβ damages lysosome, releases the lysosomal protease CTSB and activates the IL1B pathway, resulting in a microglia-dependent inflammatory response (Halle, Hornung et al. 2008). Hence, glial cells play an indispensable role in mediating neuroinflammation in neurodegenerative disorders. With the discovery of lymphatic vessels in the CNS (Louveau, Smirnov et al. 2015), there will be more emerging critical evidence of neuroinflammation mediated by non-neuronal cells in modulating the trajectory of neurodegeneration.

Although both *in vitro* and *in vivo* approaches have advanced our understanding of the molecular pathways underlying ER stress and autophagy, detailed intrinsic molecular mechanisms regulating these processes still remain to be elucidated. Taken together, available evidence suggests a key role for dysregulation of these pathways in various neurodegenerative disorders. Our knowledge is far from complete, particularly in neurons with regard to cell death/dysfunction. Given the diversity of neuronal populations

and neurodegenerative disorders, this represents a challenge. The advent and widespread applications of new imaging, gene sequencing and mouse genome engineering tools will contribute significantly to our efforts to dissect the signaling pathways underlying the UPR and autophagy both in development and disease states.

CHAPTER II

Materials and methods

3. MATERIALS AND METHODS

3.1 Reagents

Reagents: Morphine (M8777-250MG, Sigma-Aldrich), naltrexone (N3136-100MG, Sigma-Aldrich), PDGF-BB (220-BB-050, R&D), PBN (B7263-1G, Sigma-Aldrich), apocynin (sc-203321, Santa Cruz), mitoTEMPO (SML0737, Sigma-Aldrich), 4-PBA (50-230-7444, Fisher Scientific), salubrinal (SML0951, Sigma-Aldrich), wortmannin (W3144, Sigma-Aldrich), 3-MA (M9281, Sigma-Aldrich) were obtained from commercial vendors as described.

3.2 DNA constructs

The pCAGGs-IRES-eGFP plasmid has been previously described and validated (Arikkath, Israely et al. 2008). pCMV-GFP-LC3 expression vector was kindly provided from Dr. Howard Fox (Alirezaei, Kiosses et al. 2008). Both PGK-GFP-shATG7 and the PGK-GFP-control were generous gifts from Dr. Ana Maria Cuervo (Rodriguez-Muela, Koga et al. 2013).

3.3 Animals

All animal procedures were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center and the National Institutes of Health. Sprague-Dawley rats and C57BL/6N mice were purchased from Charles River Laboratories (Wilmington, MA USA). All animals were housed under constant temperature and humidity on a 12-hour light/dark cycle with

available access to food and water *ad libitum*. 2-month old male C57BL/6N mice were equally divided in two groups: saline and morphine. Morphine sulfate was dissolved in 0.9% sodium chloride (NDC 0409-4888-12, Hospira). Morphine-treated group were administrated intraperitoneal injections of morphine, thrice a day for every 8 hours, at an initial dose of 10 mg/kg with an increment of 5 mg/kg every day for 6 days. Saline groups received comparable volume of saline (as morphine group) daily. All mice were sacrificed 1 hour following the last morphine/saline injection on the last day.

3.4 Primary neuron cultures

All experimental procedures were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Primary rat hippocampal neurons were obtained from E18 rat hippocampi as previously described (Arikkath, Peng et al. 2009, Beaudoin, Lee et al. 2012). Briefly, after dissociation of the dissected brain hippocampi in Hanks buffered salt solution (14170112, Invitrogen) supplemented with 0.25% trypsin (LS003707, Worthington), primary neurons were plated at a density of 7.5×10^4 on poly-L-lysine-coated 18-mm glass coverslips. Cultures were maintained in Neurobasal medium (21103049, Invitrogen) with B27 supplement (17504044, Invitrogen), Glutamax (3505061, Invitrogen) and Penicillin-Streptomycin (15070063, Invitrogen) at 37 ℃ with 5% CO_2. Cytosine β-D-arabinofuranoside (1 μM, C1768, Sigma-Aldrich) was added to on DIV 2 and all media were replaced on DIV 3. Half of the media was replaced twice every week. Transfection was performed on DIV 7 using Lipofectamine 2000 (11668027, Invitrogen) as indicated by the manufacture. Half of the media was replaced 12 hours after transfection. Neurons (DIV 20) were treated with morphine (10 μM) for 24 hours following

one-hour pretreatment of pharmacological inhibitors. Primary hippocampal neurons were treated with PDGF-BB (20 ng/ml) for 24 hours immediately following morphine exposure.

3.5 Immunocytochemistry

For immunocytochemistry, primary rat hippocampal neurons were plated on coverslips treated with the respective agents for 24 to 48 hours, followed by fixing with 4% paraformaldehyde and 4% sucrose in PBS for 20 minutes at room temperature and permeabilization with 0.1% Triton X-100 (BP151-1, Fisher Scientific). Neurons were then incubated in 5% normal goat serum (NGS) PBS buffer for 1 hour at room temperature followed by addition of respective antibodies: PSD95 (1:500, Abcam Cat# ab18258 Lot# RRID:AB_444362), Gephyrin (1:1000, Synaptic Systems Cat# 147 011 Lot# RRID:AB_88771), or vGlut1 (1:4000, Millipore Cat# AB5905 Lot# RRID:AB_2301751) & GAD65 (1:100, DSHB Cat# GAD-6 Lot# RRID:AB_528264) overnight at 4°C. This was followed by addition of the secondary Alexa Fluor 555 goat-anti-rabbit (1:500, Thermo Fisher Scientific Cat# A27039 Lot# RRID:AB_2536100), Alexa Fluor 555 goat-anti-mouse (1:500, Thermo Fisher Scientific Cat# A-21424 Lot# RRID:AB_2535845), or Alexa fluor 488 goat anti-guinea pig (1:500, Thermo Fisher Scientific Cat# A-11073 Lot# RRID:AB_2534117) & Alexa Fluor 555 goat-anti-rabbit (1:500, Thermo Fisher Scientific Cat# A27039 Lot# RRID:AB_2536100), respectively. Cells were mounted with prolong Gold antifade reagent with DAPI (P36935, ThermoFisher).

3.6 Immunohistochemistry

Under anesthesia, animals were perfused with chilled 4% paraformaldehyde. Sections encompassing the entire hippocampus were sectioned at 12 µm on a cryostat and incubated with a blocking buffer containing 5% NGS and 0.2% Triton X-100 for 1 h at room temperature followed by addition of respective antibody pairs: vGlut1 (1:4000, Millipore Cat# AB5905 Lot# RRID:AB_2301751) & GAD65 (1:100, DSHB Cat# GAD-6 Lot# RRID:AB_528264), PSD95 (1:500, Abcam Cat# ab18258 Lot# RRID:AB_444362), Gephyrin (1:1000, Synaptic Systems Cat# 147 011 Lot# RRID:AB_88771) and incubated overnight at 4 °C. Next day, the sections were washed followed by incubation with Alexa fluor 488 goat anti-guinea pig (1:500, Thermo Fisher Scientific Cat# A-11073 Lot# RRID:AB_2534117) & Alexa Fluor 555 goat-anti-rabbit (1:500, Thermo Fisher Scientific Cat# A27039 Lot# RRID:AB_2536100), or Alexa Fluor 488 goat-anti-rabbit (1:500, Thermo Fisher Scientific Cat# A11008 Lot# RRID:AB_10563748) & Alexa Fluor 555 goat-anti-mouse (1:500, Thermo Fisher Scientific Cat# A-21424 Lot# RRID:AB_2535845), respectively, in 0.5% BSA and 0.2% Triton X-100 at RT for 2 h, followed by mounting with prolong Gold antifade reagent (P36935, ThermoFisher). Every eighth section from each mouse was used to quantify synapses using presynaptic or postsynaptic markers in the hippocampus.

3.7 Confocal microscopy & spine architecture quantitation

Imaging was performed on an inverted Zeiss LSM 700 microscope with 40X objective and a digital zoom of 4X. Contrast and brightness of overall images were consistent in each set of experiments. For primary neurons *in vitro*, the number of synapses and dendritic spines were counted manually in Zen 2010 SP1 (Carl Zeiss, CA)

using a digital zoom of 4X with Z-stack projection. These measurements were obtained on one dendrite per neuron within 80 µm from the cell body. Data was obtained from four independent cultures. The numbers of synapses and dendritic spines are indicated on the bar graph. For *in vivo*, the number of synapses in the striatum lucidum and radiatum of hippocampus were measured using Bitplane Imaris 7.6.5 software (Imaris, RRID:SCR_007370).

3.8 Western blotting

Treated cells were lysed using the Mammalian Cell Lysis kit (MCL1-1KT, Sigma-Aldrich). Equal amounts of proteins were electrophoresed in a sodium dodecyl sulphate-polyacrylamide gel (10%) under reducing conditions followed by transfer to polyvinylidene fluoride membranes. The blots were blocked with 5% bovine serum albumin in PBS. The western blots were then probed with respective antibodies recognizing the BIP (1:1000, BD Biosciences Cat# 610978 Lot# RRID:AB_398291), pPERK (1:250, Santa Cruz Biotechnology Cat# sc-32577 Lot# RRID:AB_2293243), PERK (1:250, Santa Cruz Biotechnology Cat# sc-13073 Lot# RRID:AB_2230863), IRE1α (1:500, Santa Cruz Biotechnology Cat# sc-20790 Lot# RRID:AB_2098712), ATF6 (1:1000, Abcam Cat# ab37149 Lot# RRID:AB_725571), Beclin1 (1:1000, Santa Cruz Biotechnology Cat# sc-11427 Lot# RRID:AB_2064465), ATG5 (1:1000, Novus Cat# NB110-53818 Lot# RRID:AB_828587), p62 (1:1000, Fisher Scientific Cat# 50-507-55 Lot# RRID: AB_2571590), LC3 (1:2000, Novus Cat# NB100-2220 Lot# RRID:AB_10003146) and β-actin (1:5000, Sigma-Aldrich Cat# A1978 Lot# RRID:AB_476692). The secondary antibodies were horseradish peroxidase-conjugated to goat anti mouse/rabbit IgG (1:5,000, Santa Cruz Biotechnology Cat# sc-2005 Lot# RRID:AB_631736 and Santa Cruz

Biotechnology Cat# sc-2004 Lot# RRID:AB_631746) as described previously (Yang, Yao et al. 2016).

3.9 Assessment of cellular ROS production – H₂DCFDA assays

2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA, D339, ThermoFisher) was used to measure total cellular ROS levels in hippocampal neurons according to the manufacture's recommended protocol. Neurons were seeded on either 96-well plates for fluorescence readout or on the coverslips in 12-well plates for imaging. After neurons were treated according to the experimental conditions, medium was removed and cells were incubated with 25 µM H$_2$DCFDA working solution for 30 min at 37℃. Hoechst 33342 (1.0 µM, H3570, Hoechst) was added to H$_2$DCFDA working solution during the last 5 min. Cells were subsequently rinsed in PBS and the change in H$_2$DCFDA fluorescence in 96-well plates was detected in a spectrofluorimeter set at 485-nm excitation and 530-nm emission while Hoechst 33342 was measured at 350-nm excitation and 461-nm emission. The level of intracellular ROS was indicated by the readouts of H$_2$DCFDA, and standardized according to the Hoechst 33342 levels. Neurons were mounted on coverslips and observed under an inverted Zeiss LSM 700 confocal microscope using a 40X objective.

3.10   Statistical analysis

All data are presented as mean ± SD, and were analyzed using the student's t test or ANOVA model with Dunnett's post hoc test to compare means between each group. All data were graphed and statistical analyses were performed using GraphPad Prism 6

(GraphPad Software, La Jolla, California, USA). Results were considered statistically significant if probability levels were less than 0.05.

CHAPTER III

Morphine alters the densities of excitatory and inhibitory synapses in the hippocampus

4 Morphine alters the densities of excitatory and inhibitory synapses in the hippocampus *in vivo*

Morphine abuse is associated with cognitive changes that are likely linked to synaptic alterations. We used an *in vivo* model to examine if morphine administration altered the densities of excitatory and inhibitory synapses in the hippocampus, a key center for learning and memory. Groups of mice were exposed to either saline or morphine with intraperitoneal injection thrice a day, starting at an initial dose of 10 mg/kg followed by ramping the dose by 5mg/kg per day for six consecutive days (Pal and Das 2013). At the end of the treatment, mice were sacrificed and the hippocampus was subjected to immunostaining and confocal microscopy. Morphine administration resulted in significant decrease of density of excitatory synapses with a significant enhancement of density of inhibitory synapses in the stratum lucidum and radiatum areas of the hippocampus, as indicated by densities of presynaptic (vesicular glutamate transporter 1 (vGlut1) and glutamic acid decarboxylase 65 (GAD65) **(Figures 4.1A & 4.1B)** and postsynaptic markers (postsynaptic density protein 95 (PSD95) and gephyrin) **(Figures 4.2A & 4.2B)** for excitatory and inhibitory synapses, respectively. Morphine administration thus induces a decrease in the density of excitatory synapses with an increase in the density of inhibitory synapses in the hippocampus *in vivo*.

Figure 4.1 Morphine alters the presynaptic densities of excitatory and inhibitory synapses in the hippocampus *in vivo.*

(A) Representative confocal images of the stratum lucidum and radiatum regions of mouse hippocampi were stained with presynaptic protein vGlut1 and GAD65, and (B) quantification of vGlut1 and GAD65 puncta (scale bar = 10 μm, n=6/group; Student's t test). All data are presented as mean ± SD, ** P<0.01 vs saline group.

Figure 4.2 Morphine alters the postsynaptic densities of excitatory and inhibitory synapses in the hippocampus *in vivo.*

(A) Representative confocal images of the stratum lucidum and radiatum regions of mouse hippocampi stained with postsynaptic protein PSD95 and gephyrin, and (B) quantification of PSD95 and gephyrin puncta (scale bar = 10 μm, n=6/group; Student's t test). All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs saline group.

5   Morphine alters the densities of excitatory and inhibitory synapses in the hippocampus *in vitro*

We next sought to examine if we could recapitulate morphine mediated synaptic alterations in an *in vitro* dissociated neuronal cell culture system (Liao, Grigoriants et al. 2007, Nassirpour, Bahima et al. 2010, Beaudoin, Lee et al. 2012, Miller, Zhang et al. 2012). There are two advantages to this approach. Since the primary culture model is predominantly neuronal in nature, if we observed synaptic alterations with morphine, it would indicate that these effects are mediated by a direct effect on neurons, as opposed to a secondary consequence of activation of other cell types in the hippocampus, including microglia and astrocytes (Horvath, Romero-Sandoval et al. 2010, Vacca, Marinelli et al. 2013) In addition, this is a well-established tractable model that would allow the exploration of underlying mechanisms.

We exposed GFP-transfected rat primary hippocampal neurons at DIV 20 to morphine (10 μM) for 24 hours followed by immunostaining for presynaptic and postsynaptic markers for both excitatory and inhibitory synapses. The neurons were subjected to confocal microscopy and synapse densities for both types of synapses were quantitated. Similar to our *in vivo* findings morphine exposure resulted in a significant loss in the density of both dendritic spines and excitatory synapses **(Figures 5.1A & 5.1C)** and enhanced the density of inhibitory synapses **(Figures 5.1B & 5.1C)**. Morphine exposure promoted a decrease in the density of excitatory synapses with an increase in the density of inhibitory synapses *in vitro*. Taken together, the *in vivo* and *in vitro* data indicate that morphine regulates density of excitatory and inhibitory synapses and, that these effects are mediated by its direct action on neurons.
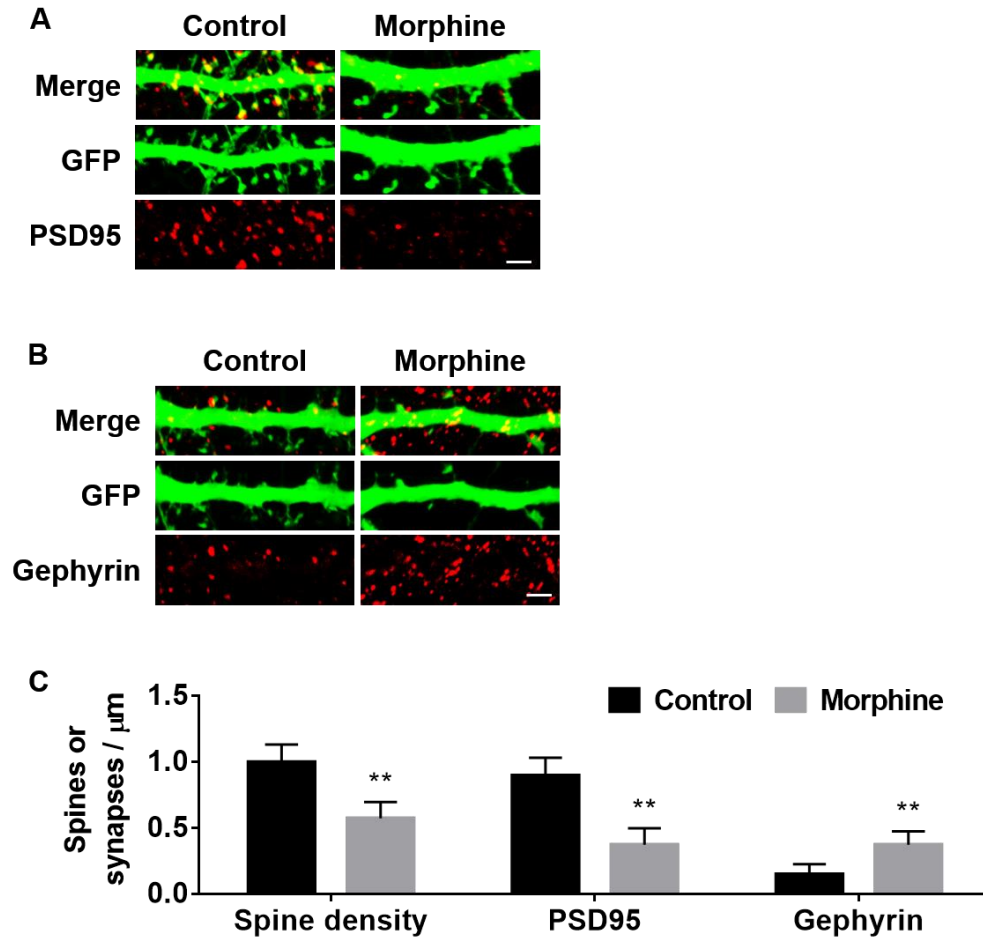
Figure 5.1 Morphine alters the postsynaptic densities of excitatory and inhibitory synapses in the hippocampus *in vitro*.

(A) Representative confocal images of GFP-transfected primary rat hippocampal neurons (DIV 20) exposed to morphine (10 µM) for 24 h and immunostained for PSD95 (scale bar = 5 µm). (B) Representative confocal images of GFP-transfected primary rat hippocampal neurons treated with morphine and immunostained for gephyrin (scale bar = 5 µm). (C) Quantification of spine density, excitatory and inhibitory synapses of Figures 5.1A & 5.1B (Student's t test). Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, ** $P < 0.01$ vs control group.

To confirm that these effects on synapses are specifically mediated by morphine mediated activation of the MOR we pretreated neurons with the opioid receptor antagonist naltrexone (10 µM) (Yue, Tumati et al. 2008, Mohan, Davis et al. 2010) one hour prior to morphine treatment before subjecting the neurons to immunostaining with synaptic markers, confocal microscopy and quantitation of synaptic densities. Pretreatment of neurons with naltrexone significantly abrogated morphine-mediated loss of dendritic spines and excitatory synapses, while suppressing upregulation of inhibitory synapses **(Figures 5.2A & 5.2B)**. The effects of morphine on synaptic densities are thus mediated by its ability to bind and activate the MOR.
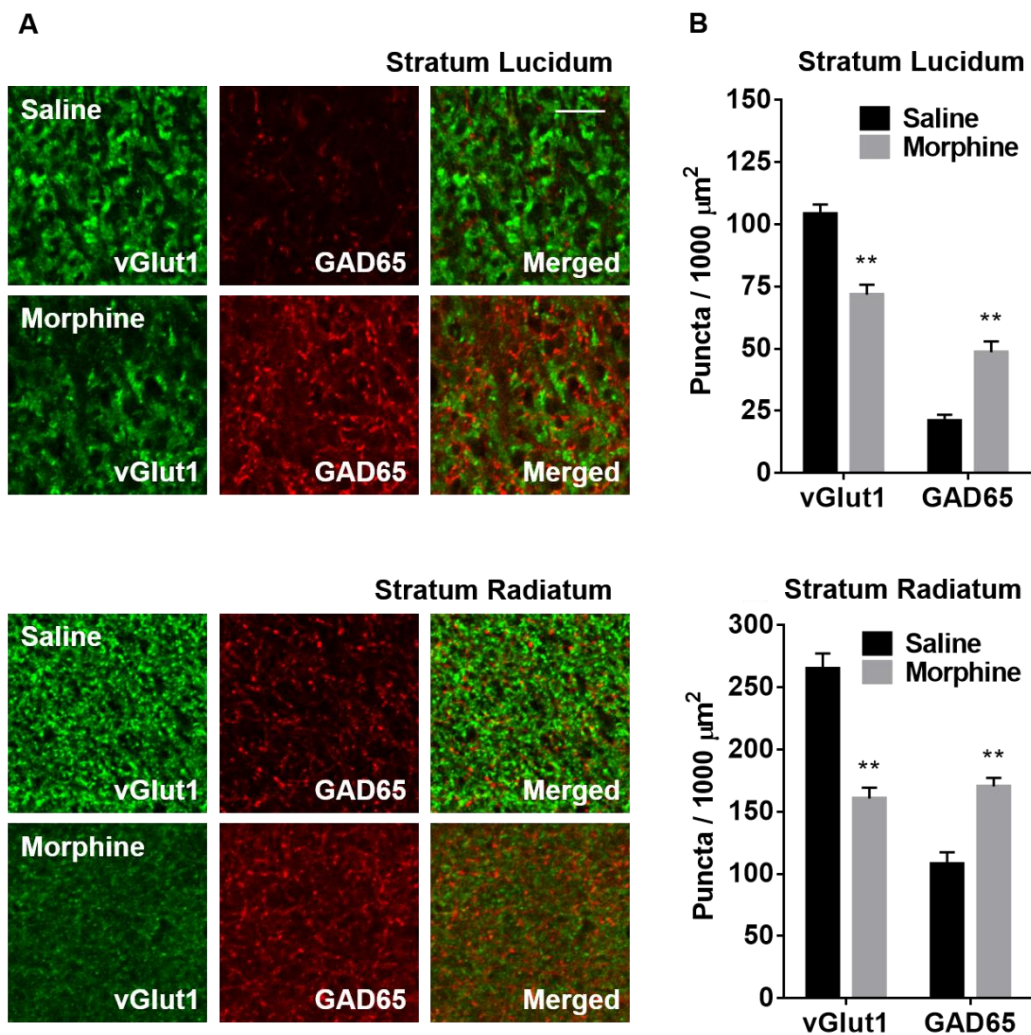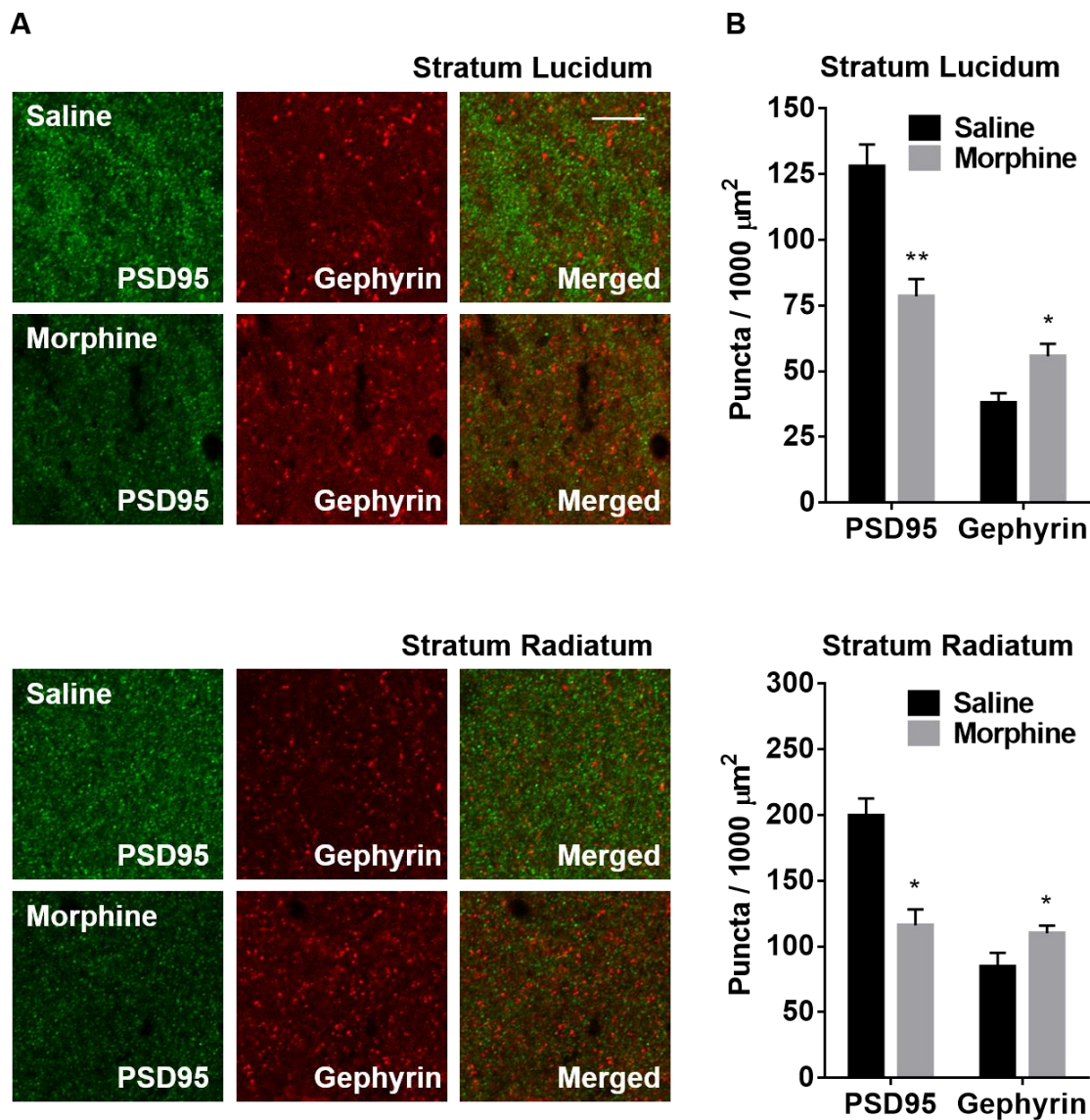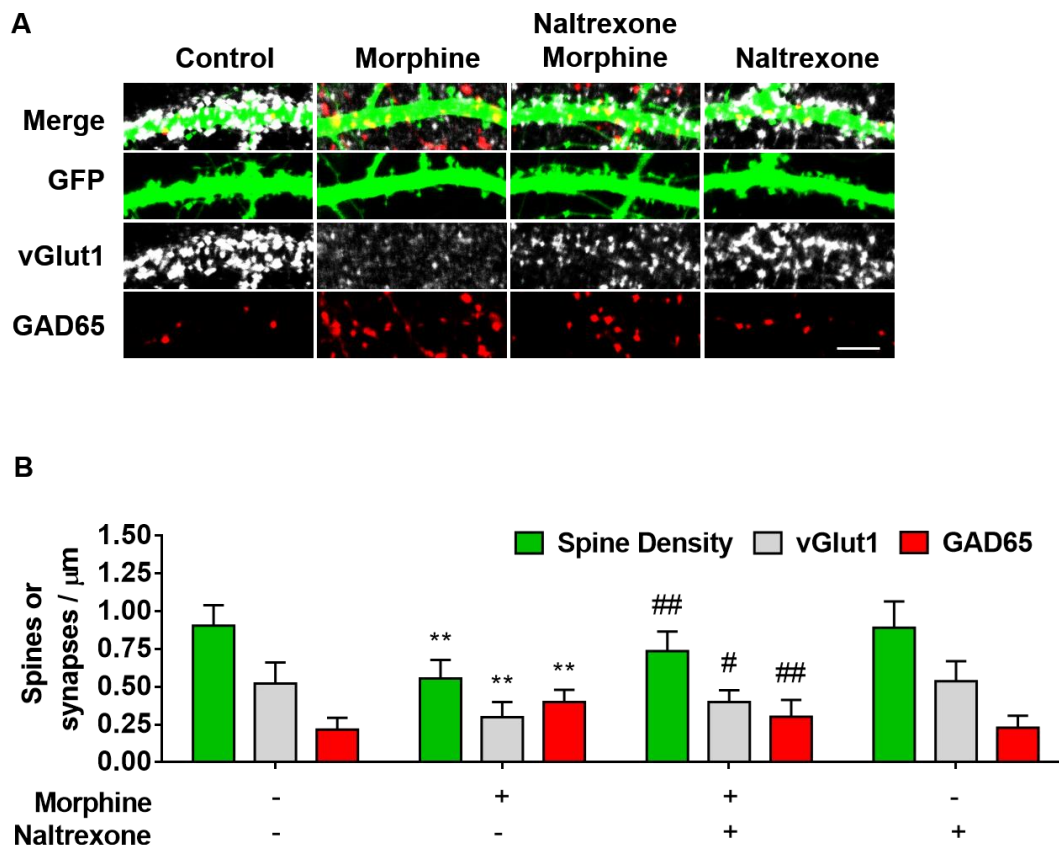
Figure 5.2 Morphine alters the presynaptic densities of excitatory and inhibitory synapses in the hippocampus *in vitro*.

(A) Representative confocal images of GFP-transfected primary rat hippocampal neurons treated with naltrexone (10 μM) for 1 h followed by morphine for 24 h, stained with vGlut1 and GAD65 and (B) quantifications of spine density, excitatory and inhibitory synapses (scale bar = 5 μm, one-Way ANOVA). Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, ** $P<0.01$ vs control/saline group; # $P<0.05$, ## $P<0.01$ vs morphine group.

6 Conversation

The hippocampus, an integrative center for regulating learning and memory and addiction (Koob and Volkow 2010, Moron and Green 2010), is also one of the brain regions with significant expression of mu opioid receptor (MOR) (Le Merrer, Becker et al. 2009), suggesting that the effects of opioids on cognition could be mediated by signaling via the MOR in the hippocampus. Alterations in synaptic density, structure, function and plasticity have been identified as key components of the finely tuned machinery underlying cognition in the hippocampus (Marder and Goaillard 2006, Zhong and Gerges 2010). Such mechanisms operate at both excitatory and inhibitory synapses (Bateup, Johnson et al. 2013, Calfa, Li et al. 2015, Nelson and Valakh 2015, Villa, Berry et al. 2016).

Neural circuits are regulated by activity dependent feedback that tightly control neural excitability. Deficits or imbalances in excitation and inhibition can have serious consequences that contribute to pathological conditions. Such perturbations are frequently observed in neurodevelopmental disorders associated with cognitive disabilities (Sandi and Haller 2015).

Both *in vitro* and *in vivo* studies have demonstrated that morphine regulates excitatory spine density in the hippocampus (Robinson, Gorny et al. 2002, Liao, Lin et al. 2005, Liao, Grigoriants et al. 2007, Zheng, Zeng et al. 2010). A profound loss in dendritic spines in the hippocampal CA1 was observed in rats exposed to long-term administration of morphine (Robinson, Gorny et al. 2002). Consistent with this finding, studies also indicate a reduction in spine density in cultured hippocampal neurons treated with morphine *in vitro* (Liao, Lin et al. 2005, Liao, Grigoriants et al. 2007, Zheng, Zeng et al. 2010). However, our knowledge of the molecular mechanisms that regulate the ability of morphine to regulate spines and excitatory synapses remains far from complete. The

effects of morphine on inhibitory synapses remain unclear. The balance of excitatory and inhibitory synapses is crucial for maintenance of normal functioning of neural circuits (Froemke, Merzenich et al. 2007) and higher order brain functions including cognition. There are limited avenues for therapeutic intervention to address cognitive deficits resulting from long-term use of morphine. Our data indicated that morphine mediated alterations in the balance of excitatory and inhibitory synapses contribute to deficits in the neural circuits underlying cognition, which is thus of critical importance in our understanding of how neural circuitry responds to opioids and could provide insights into the development of therapeutic targets aimed at restoring normal cognitive function in morphine users.

CHAPTER IV

The role of oxidative stress in morphine-mediated synaptic alterations

7   Morphine induces oxidative stress in hippocampal neurons

We sought to examine the components of the cellular signaling pathway that allow morphine to regulate synapses. Morphine has been shown to induce oxidative stress via generation of reactive oxygen species (ROS) (Skrabalova, Drastichova et al. 2013) in both macrophages as well as *in vivo* in the spinal cord (Bhat, Bhaskaran et al. 2004, Malik, Khalique et al. 2011, Podhaizer, Zou et al. 2012). We next examined whether exposure of hippocampal neurons to morphine resulted in increased production of ROS and also sought to identify the intracellular source of ROS.

We took advantage of the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescence assay (Gomes, Fernandes et al. 2005) to measure ROS levels in primary rat neurons exposed to morphine. Morphine promoted ROS production in a time-dependent manner with the peak response in about an hour post exposure **(Figures 7A & 7B)**.
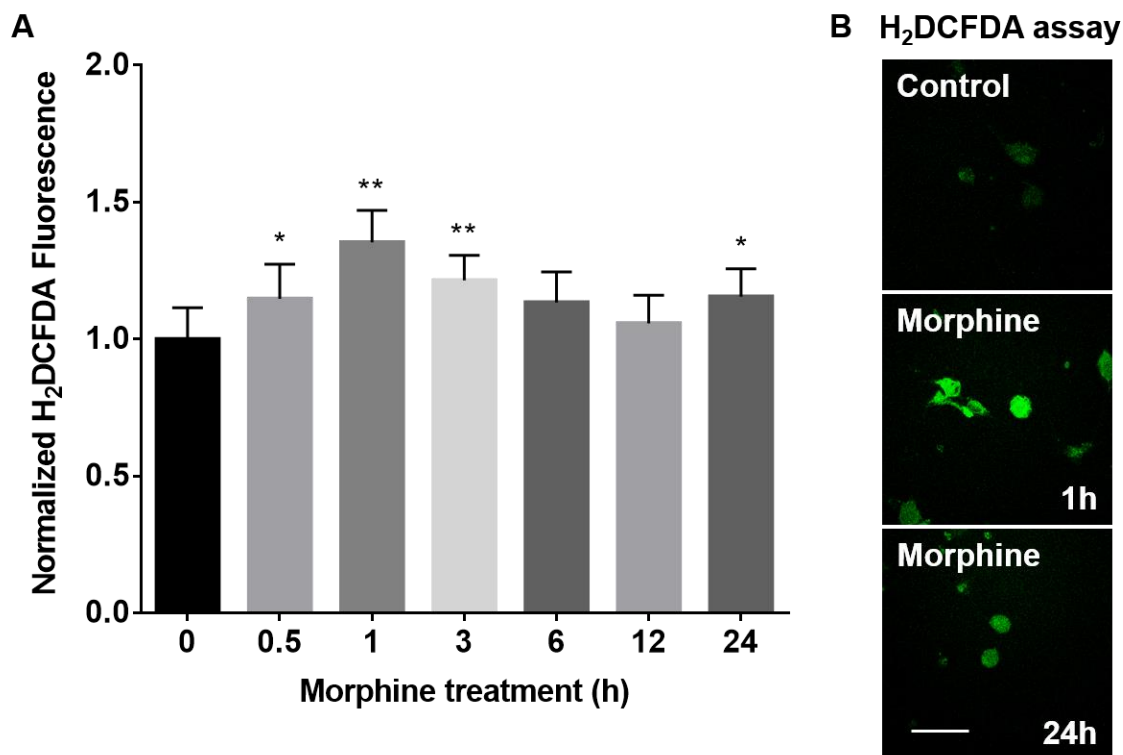
Figure 7 Morphine induces oxidative stress in hippocampal neurons.

(A) Time course of ROS generation in primary hippocampal neurons exposed to morphine by H_2DCFDA assay. (B) Representative H_2DCFDA assay images of neurons at 1 h and 24 h post morphine exposure (scale bar = 40 μm). Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control group using one-Way ANOVA analysis.

8 Morphine induces ROS production via NADPH oxidase in hippocampal neurons

Mitochondria and NADPH oxidases are the two major intracellular sources of ROS (Dikalov 2011, Luo 2014, Xie, Hou et al. 2016). To determine the major contributing source of ROS following morphine exposure, neurons were pretreated with pharmacological inhibitors specific for either mitochondrial or NADPH oxidase ROS, exposed to morphine and investigated for levels of ROS generated using the H_2DCFDA fluorescence assay. Pretreatment of cells with either naltrexone (MOR antagonist), ROS scavenger phenyl-N-tert-butylnitrone (PBN) or inhibitor of NADPH oxidase - apocynin, significantly suppressed morphine-mediated induction of ROS generation **(Figure 8)**. Mitochondria-targeted antioxidant MitoTEMPO however, failed to inhibit morphine-mediated induction of oxidative stress **(Figure 8)**. These results are thus consistent with a role for NADPH oxidase, but not that of mitochondria, in generating ROS in hippocampal neurons exposed to morphine.

A
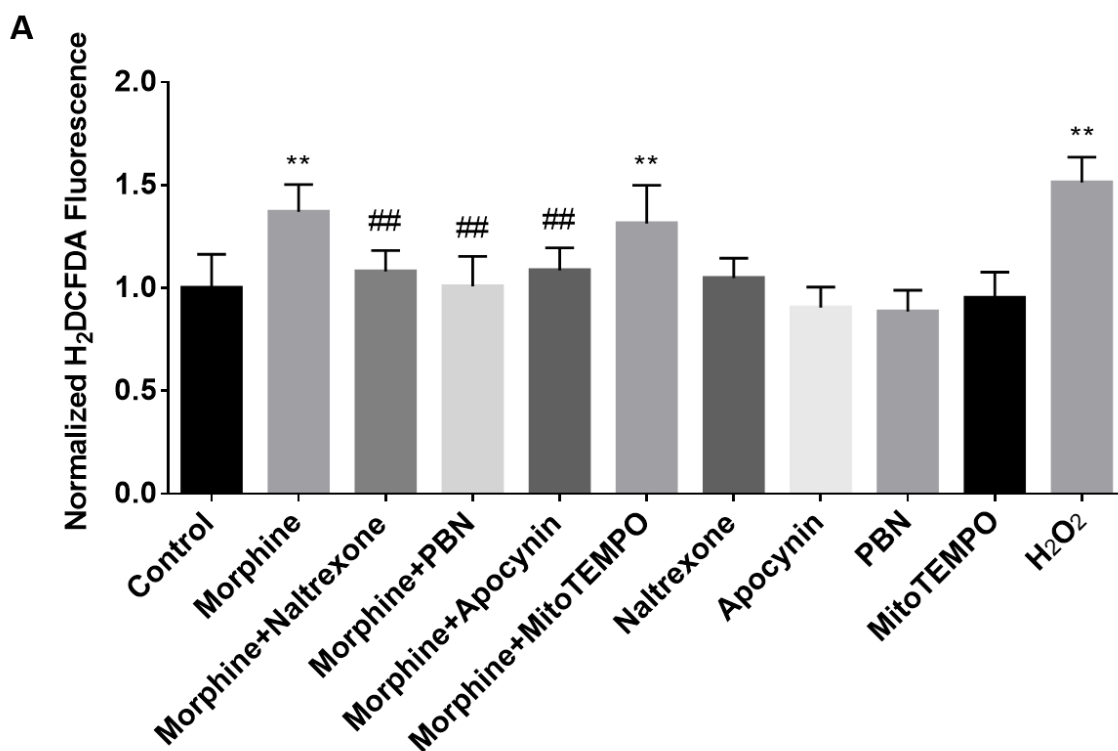
Figure 8 Morphine induces ROS production via NADPH oxidase in hippocampal neurons.

(A) Neurons were pretreated with naltrexone, PBN (500 μM), apocynin (10 μM) or MitoTEMPO (100 μM) for half an hour followed by one-hour morphine treatment and assessed for ROS generation by H_2DCFDA assay. Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, ** $P<0.01$ vs control group; ## $P<0.01$ vs morphine group using one-Way ANOVA analysis.

9   The role of oxidative stress in morphine-mediated synaptic alterations

To examine the relevance of morphine and NADPH oxidase-mediated ROS in regulating synapses, we performed experiments similar to that described for **Figure 5.2**, in neurons pretreated with the NADPH oxidase inhibitor apocynin (10 µM). Pretreatment of cells with apocynin resulted in inhibition of morphine-mediated synaptic alterations, including downregulation of both spine density and excitatory synapses with a concomitant upregulation of inhibitory synapses **(Figures 9A & 9B)**. These data are consistent with a model in which morphine-mediated ROS generation, via the MOR, influences densities of excitatory and inhibitory synapses in hippocampal neurons.
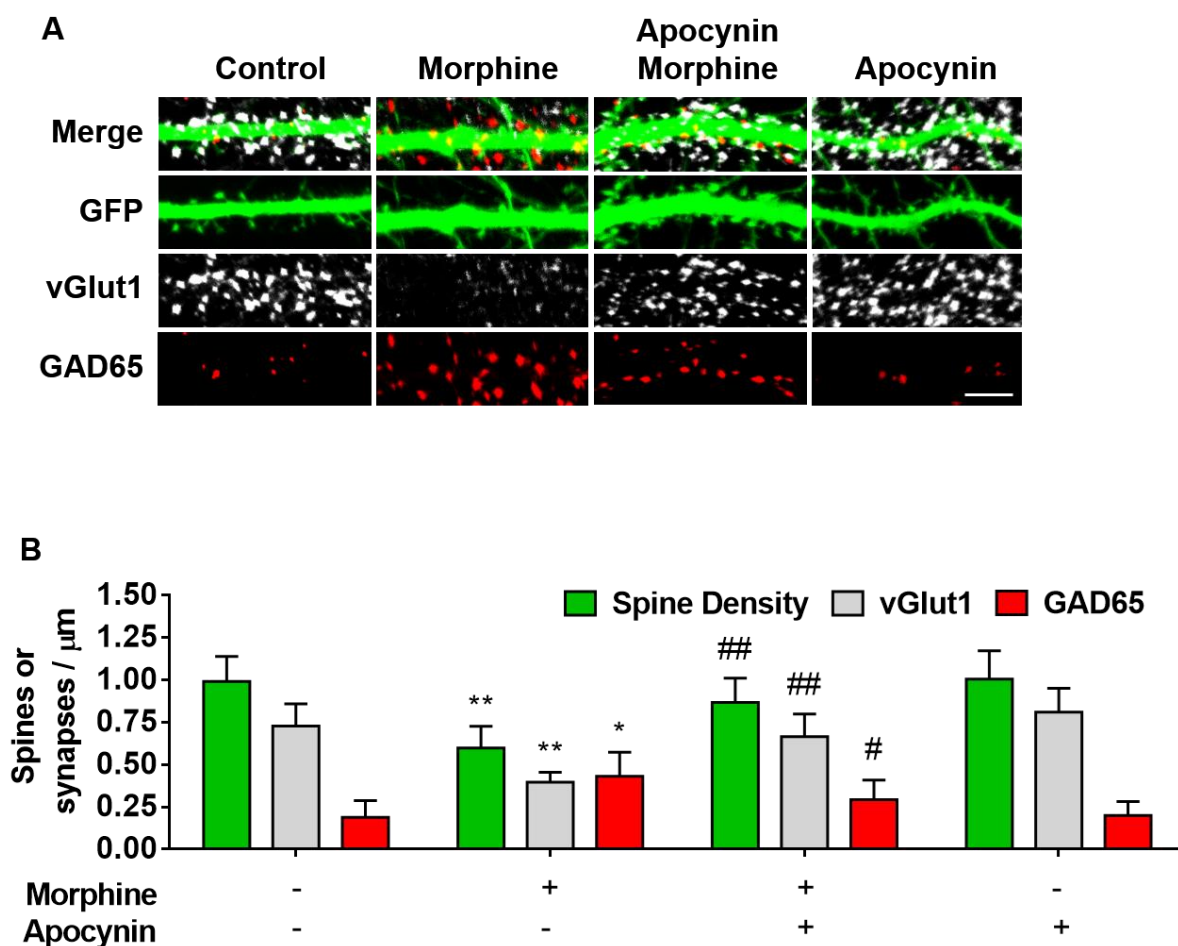
Figure I The role of oxidative stress in morphine-mediated synaptic alterations.

(A) Representative confocal images of GFP-transfected primary rat hippocampal neurons treated with apocynin for 1 h followed by morphine for 24 h stained with vGlut1 and GAD65 antibodies and (B) quantifications of spine density, excitatory and inhibitory synapses (scale bar = 5 μm). Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control group; # $P<0.05$, ## $P<0.01$ vs morphine group using one-Way ANOVA analysis.

10 Discussion

The highly metabolic nature of the brain with high oxygen consumption results in the robust production of ROS in neurons. The levels of ROS are tightly maintained through the activity of a network of antioxidants. At physiologically relevant concentrations, ROS has been demonstrated to participate in cellular mechanisms that underlie synaptic plasticity and hence cognitive functions (Massaad and Klann 2011). Excessive levels of ROS are also associated with decreases in cognitive performance. The ability of morphine to elicit alterations in the level of ROS is consistent with data from other cells such as human neuroblastoma, murine macrophages and microglia (Bhat, Bhaskaran et al. 2004, Ma, Yuan et al. 2015, Cai, Kong et al. 2016).

While several sources for the generation of ROS exist in neurons (Brennan, Suh et al. 2009, Shadel and Horvath 2015), our data indicate that morphine activation of the MOR specifically harnesses the NADPH oxidase pathway for ROS generation, which in turn, activates a specific downstream signaling pathway to promote synaptic changes. Targeting NADPH oxidase mediated ROS generation could thus be envisioned as a viable target to ameliorate synaptic alterations induced by morphine.

CHAPTER V

The role of ER stress in morphine-mediated synaptic alterations

11 Morphine induces ER stress in hippocampal neurons

We examined downstream mechanisms that link morphine induced ROS generation to synaptic alterations. To this end, we focused on the ER stress pathway. The cellular redox status altered by ROS generation has been reported to affect ER protein folding, resulting in ER stress (Guo, Liao et al. 2015, Wang, Yu et al. 2015, Arimoto-Matsuzaki, Saito et al. 2016). In response to ER stress, there is an initiation of the unfolded protein response (UPR) that is induced as an adaptive mechanism, involving upregulation of the three cascading sensors: protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), inositol-requiring protein 1 α (IRE1α) and activating transcription factor 6 (ATF6), and the ER chaperone binding immunoglobulin protein (BIP) (Hetz and Mollereau 2014, Binet and Sapieha 2015). Although ER stress triggered by oxidative stress has been suggested to play a crucial role in neurodegenerative diseases (Lindholm, Wootz et al. 2006, Uehara, Nakamura et al. 2006, Chaudhari, Talwar et al. 2014), its effects in synaptic structure and function remain unclear.

To examine whether morphine exposure could induce ER stress, we investigated the expression of several critical UPR mediators BIP, pPERK, IRE1α and ATF6 in the hippocampus of mice exposed to morphine. Hippocampal lysates from mice with morphine injection were assessed for expression of UPR mediators by western blot analyses. As shown in **Figures 11.1A & 11.1B**, there was a significant increase of the major UPR mediators in the hippocampal lysates of morphine-injected mice compared with the saline-injected controls.
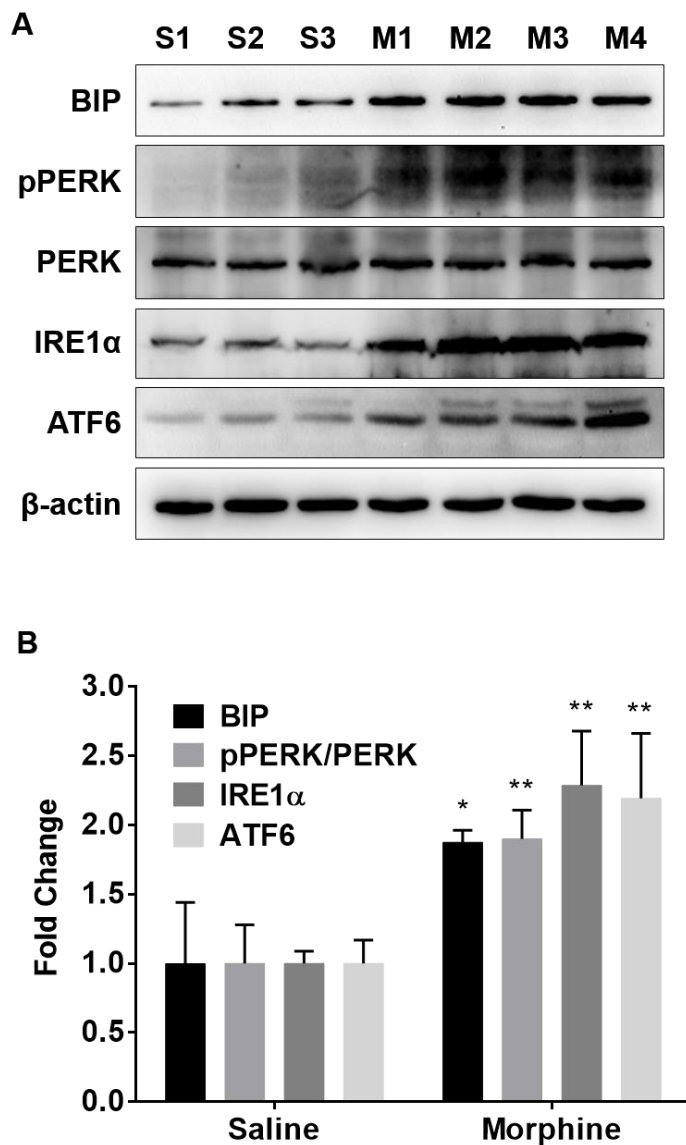
Figure 11.1 Morphine induces ER stress in hippocampus *in vivo.*

     (A) Representative western blot and (B) quantification of ER stress mediators (BIP, pPERK, IRE1α and ATF6) in the hippocampal lysates from mouse injected with saline or morphine (n=3-4/group). Quantification of western blot results were all normalized to β-actin except that pPERK was normalized to PERK. All data are presented as mean ± SD, * P<0.05, ** P<0.01 vs saline group using Student's t test.

In addition, we found a time-dependent increase of BIP in the primary hippocampal neurons in the presence of morphine, indicating that morphine also triggered ER stress *in vitro,* consistent with our *in vivo* findings **(Figures 11.2A & 11.2B)**. To confirm that these alterations are a direct consequence of the activation of MOR by morphine, we pretreated primary neurons in culture with the MOR antagonist naltrexone prior to exposure with morphine and examined the expression of ER stress markers by western blot analyses. Pretreatment of neurons with naltrexone inhibited morphine-mediated increase of ER stress markers **(Figures 11.3A & 11.3B).** These studies thus indicated that morphine mediated activation of the MOR promotes ER stress and initiates the UPR response, both *in vivo* and *in vitro*.

A

Morphine (h)   0   1   3   6   12   24   48

BIP

β-actin

B
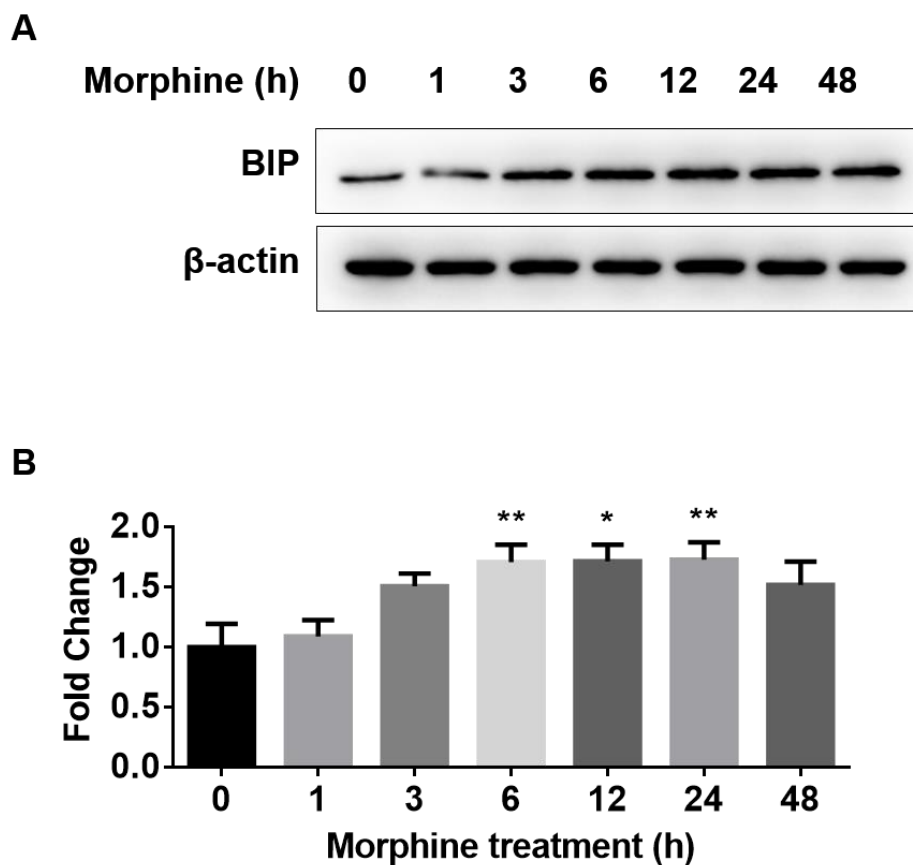
Figure 11.2 Morphine induces upregulation of BIP in a time-dependent manner in hippocampal neurons.

(A) Representative time course western blot and (B) quantification of BIP in primary neurons exposed to morphine. Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control group using one-Way ANOVA analysis.
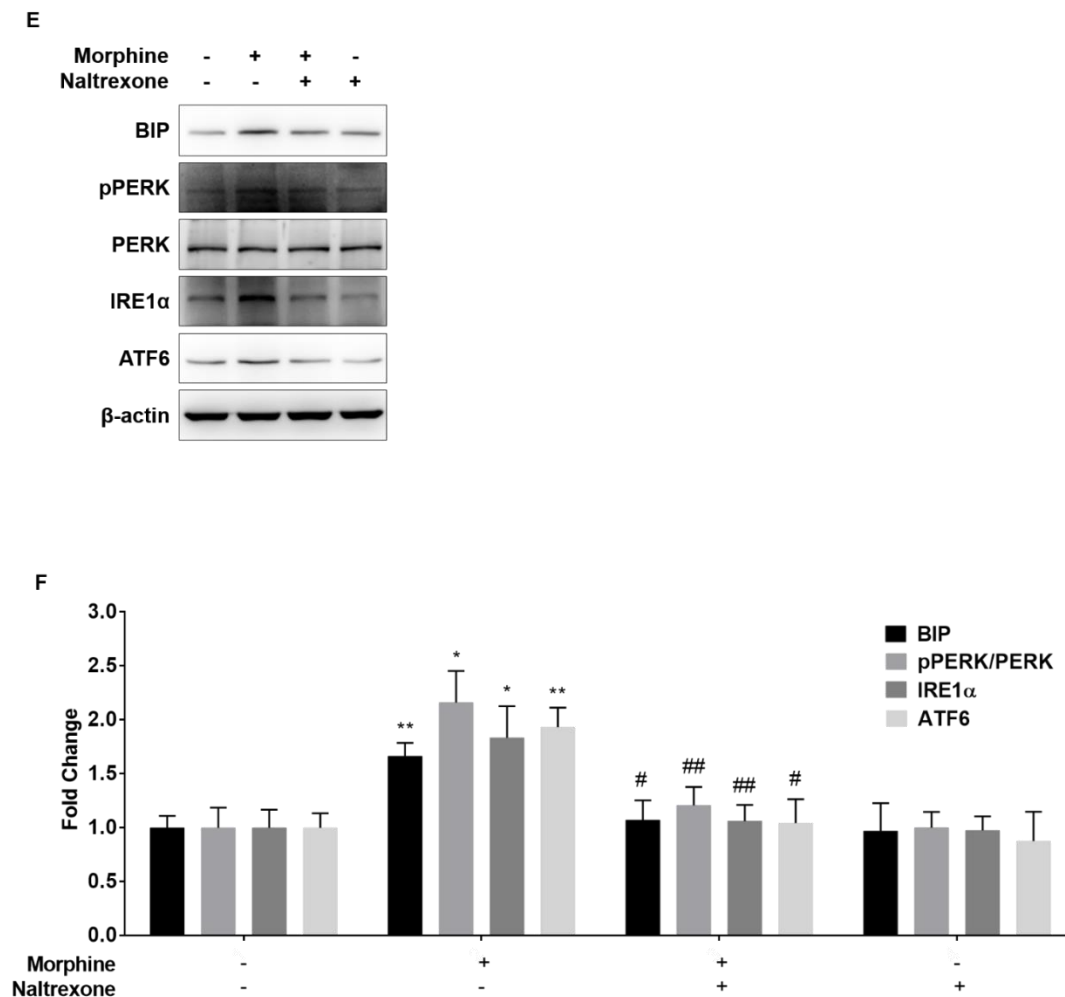
Figure 11.3 Morphine induces ER stress in hippocampus *in vitro*.

(A) Representative western blot and (B) quantification of BIP, pPERK, IRE1α and ATF6 in the cell lysates from neurons pretreated with naltrexone for 1 h followed by 24-hour morphine treatment. Quantification of western blot results were all normalized to β-actin except that pPERK was normalized to PERK. Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, * P<0.05, ** P<0.01 vs control group; # P<0.05, ## P<0.01 vs morphine using one-Way ANOVA analysis.

12 The role of ER stress in morphine-mediated synaptic alterations

Since morphine mediates ER stress in hippocampal neurons both *in vitro* and *in vivo*, we sought to assess the role of ER stress in morphine-mediated synaptic alterations. Pharmacological blocking of ER stress by 4-phenylbutyrate (4-PBA, a chaperone-like ER stress inhibitor) in the primary rat hippocampal neurons resulted in attenuation of morphine-mediated induction of synaptic alterations **(Figures 12A & 12B).**
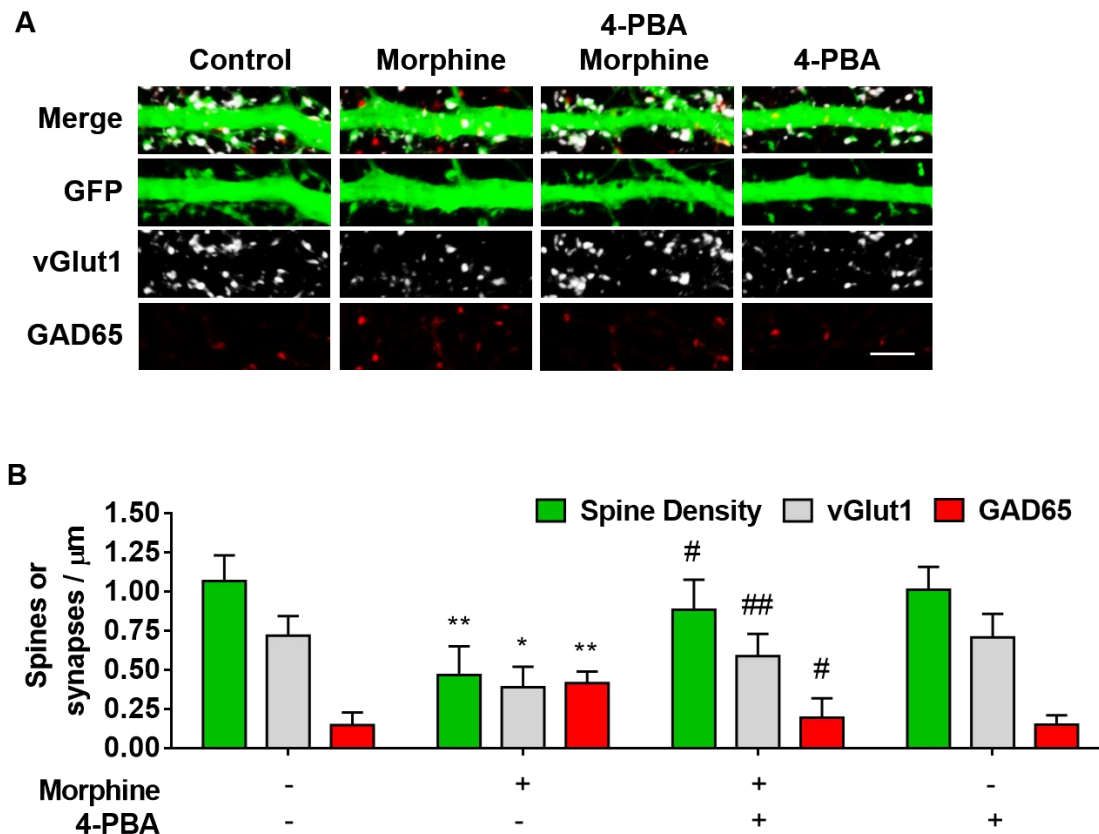
Figure 12 The role of ER stress in morphine-mediated synaptic alterations.

(A) Representative confocal images of GFP-transfected primary rat hippocampal neurons treated with 4-PBA (1 mM) for 1 h followed by morphine for 24 h, stained with vGlut1 and GAD65 and (B) quantifications of spine density, excitatory and inhibitory synapses (scale bar = 5 μm). Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control group; # $P<0.05$, ## $P<0.01$ vs morphine using one-Way ANOVA analysis.

13 The role of oxidative stress in morphine-mediated ER stress

We next explored the link between morphine-mediated induction of ER stress and oxidative stress. Morphine-mediated increase of ER stress regulators was ameliorated in hippocampal neurons pretreated with oxidative stress inhibitors PBN or apocynin **(Figures 13.1A & 13.1B)**.

On the other hand, in neurons pretreated with the ER stress inhibitor, 4-PBA, there was no change in morphine-mediated induction of ROS **(Figure 13.2)**. These results are consistent with a model in which morphine mediated oxidative stress functions upstream of ER stress to regulate synaptic densities in hippocampal neurons.

I

Morphine	-	+	+	+	-	-
PBN	-	-	+	-	+	-
Apocynin	-	-	-	+	-	+

BIP

pPERK

PERK

IRE1α

ATF6

β-actin

J
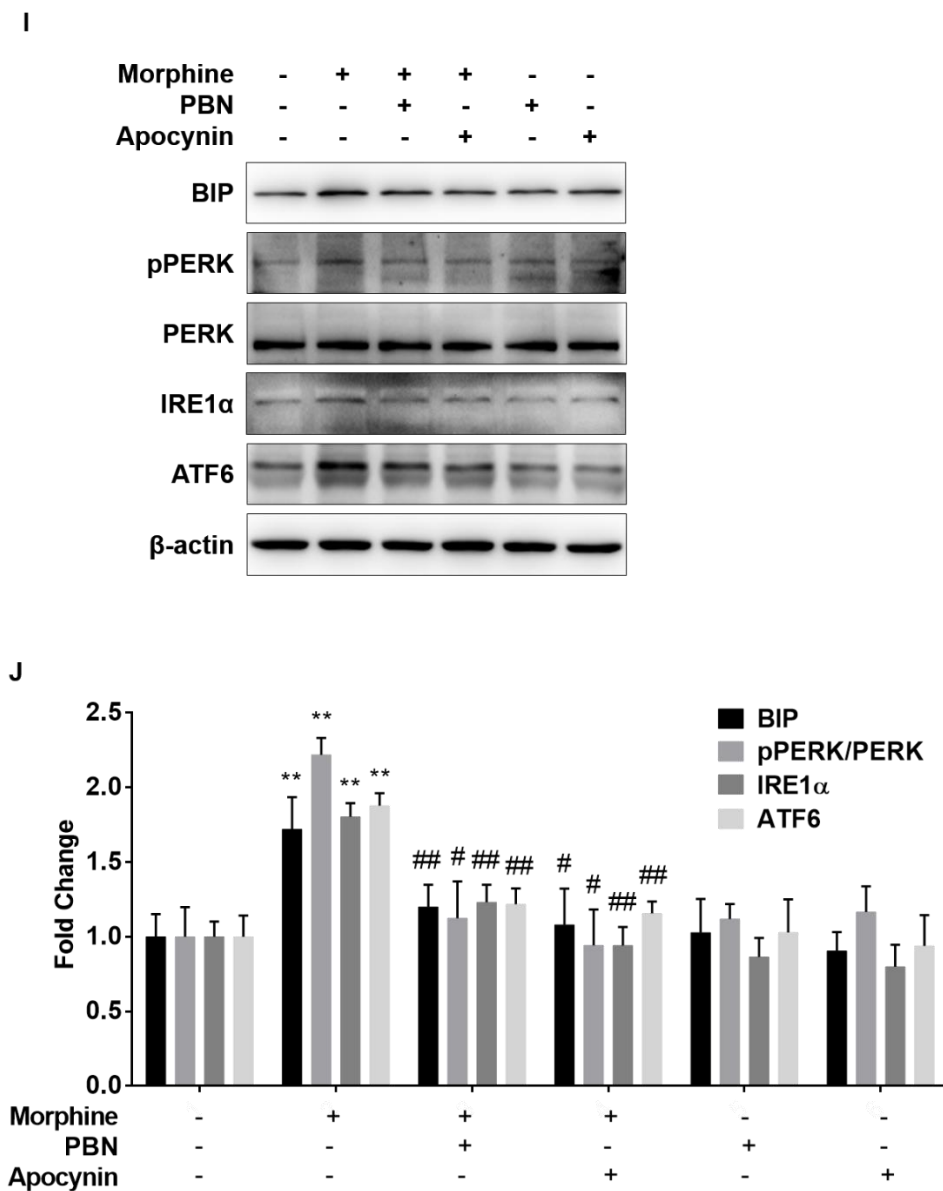
Figure 13.1 The role of oxidative stress in morphine-mediated ER stress.

(A) Representative western blot and (B) quantification of BIP, pPERK, IRE1α and ATF6 in the cell lysates from neurons pretreated with PBN or apocynin for 1 h followed by 24-hour morphine treatment. Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control group; # $P<0.05$, ## $P<0.01$ vs morphine group using one-Way ANOVA analysis.

A
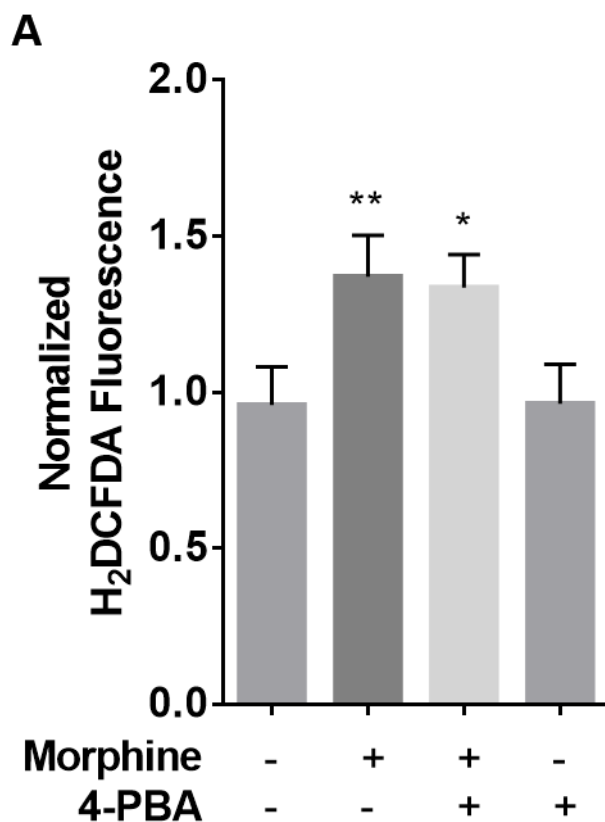
Figure 13.2 The role of ER stress in morphine-mediated oxidative stress

(A) H_2DCFDA assay of neurons pretreated with 4-PBA for half an hour followed by one-hour morphine treatment. Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control group; # $P<0.05$, ## $P<0.01$ vs morphine using one-Way ANOVA analysis.

14 Discussion

The ER is a subcellular compartment that governs protein quality control in the secretory pathway to prevent protein aggregation and misfolding (Cai, Arikkath et al. 2016). Activation of ER stress promotes the unfolded protein response (UPR) to reestablish homoeostasis. While the role of these pathways in relation to neurodegenerative disorders has been explored (Hetz and Mollereau 2014), it remains unclear how ER stress independent of cell death, contributes to other neuronal processes including synaptic alterations. Although ER stress (Huang, Hu et al. 2015) and components of the UPR response have been linked to deficits in learning and memory (Martinez, Vidal et al. 2016), the underlying mechanisms remain incompletely understood.

Our study provides the first direct link between morphine and ER stress and indicates that morphine activation via the MOR and ROS mediated signaling promotes upregulation of both ER chaperone protein BIP and the three major arms of ER stress (pPERK, IRE1α and ATF6), that eventually regulate both excitatory and inhibitory synapses. Interestingly, pretreatment of neurons with a chaperone-like ER stress inhibitor 4-PBA significantly abrogated morphine-mediated synaptic loss, suggesting another viable target for therapeutic intervention in morphine-mediated cognitive decline.

CHAPTER VI

The role of autophagy in morphine-mediated synaptic alterations

15 Morphine induces autophagy in hippocampal neurons

We have previously demonstrated that cocaine mediated induction of both ER stress as well as downstream autophagy to play a key role in microglial activation (Guo, Liao et al. 2015). Autophagy is an intracellular catabolic process that delivers intracellular organelles or misfolded proteins for lysosomal degradation (Cai, Arikkath et al. 2016). Previous studies have demonstrated that morphine exposure leads to enhanced autophagic activity in both human neuroblastoma cell line as well as in the rat hippocampus (Zhao, Zhu et al. 2010). Interestingly, neuronal autophagy has also been reported to play a key role in synaptic pruning (Tang, Gudsnuk et al. 2014).

Next we examined whether morphine mediated synaptic alterations involved upregulation of autophagy. Western blot analyses of hippocampal lysates from mice administrated morphine demonstrated that morphine exposure also resulted in induction of autophagy mediators including Beclin1, ATG5 and LC3-II with a concomitant downregulation of the autophagy substrate protein p62 **(Figures 15.1A & 15.1B).** Consistent with our *in vivo* findings, exposure of primary hippocampal neurons to morphine resulted in increased expression of LC3-II in a time-dependent manner **(Figures 15.2A & 15.2B).** We examined the number of autophagosomes in primary neurons transfected with GFP-LC3 plasmids treated with morphine. As shown in **Figures 15.3A & 15.3B**, morphine exposure resulted in significant increase in the number of GFP-LC3 puncta. Morphine exposure also upregulated expression of Beclin1, ATG5 and LC3-II with a decrease in p62 levels in primary hippocampal neurons as examined by western blot analyses, and this effect of morphine was reversed in cells pretreated with MOR antagonist naltrexone **(Figures 15.4A & 15.4B).**

A

B
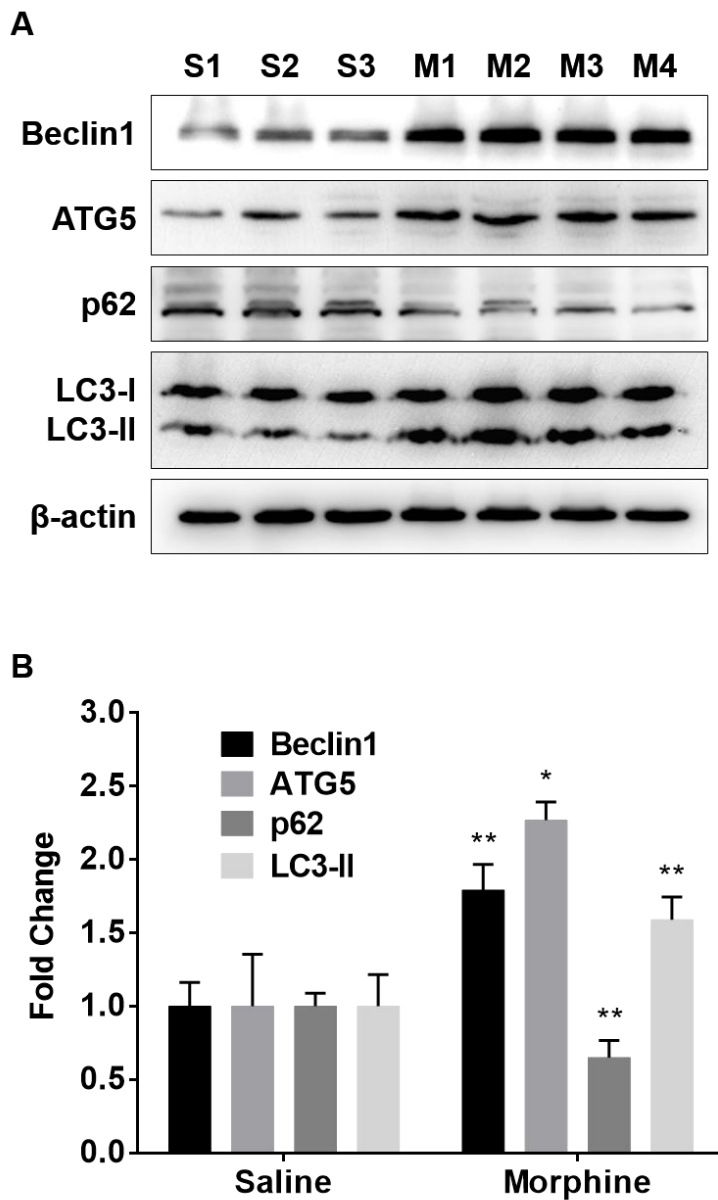
Figure 15.1 Morphine induces autophagy in hippocampus *in vivo.*

(A) Representative western blot and (B) quantification of autophagy mediators (Beclin1, ATG5, p62 and LC3-II) in the hippocampal lysates from mouse injected with saline or morphine (n=3-4/group; * P<0.05, ** P<0.01 vs saline group using Student's t test). Quantification of western blot results were all normalized to β-actin.

A

B
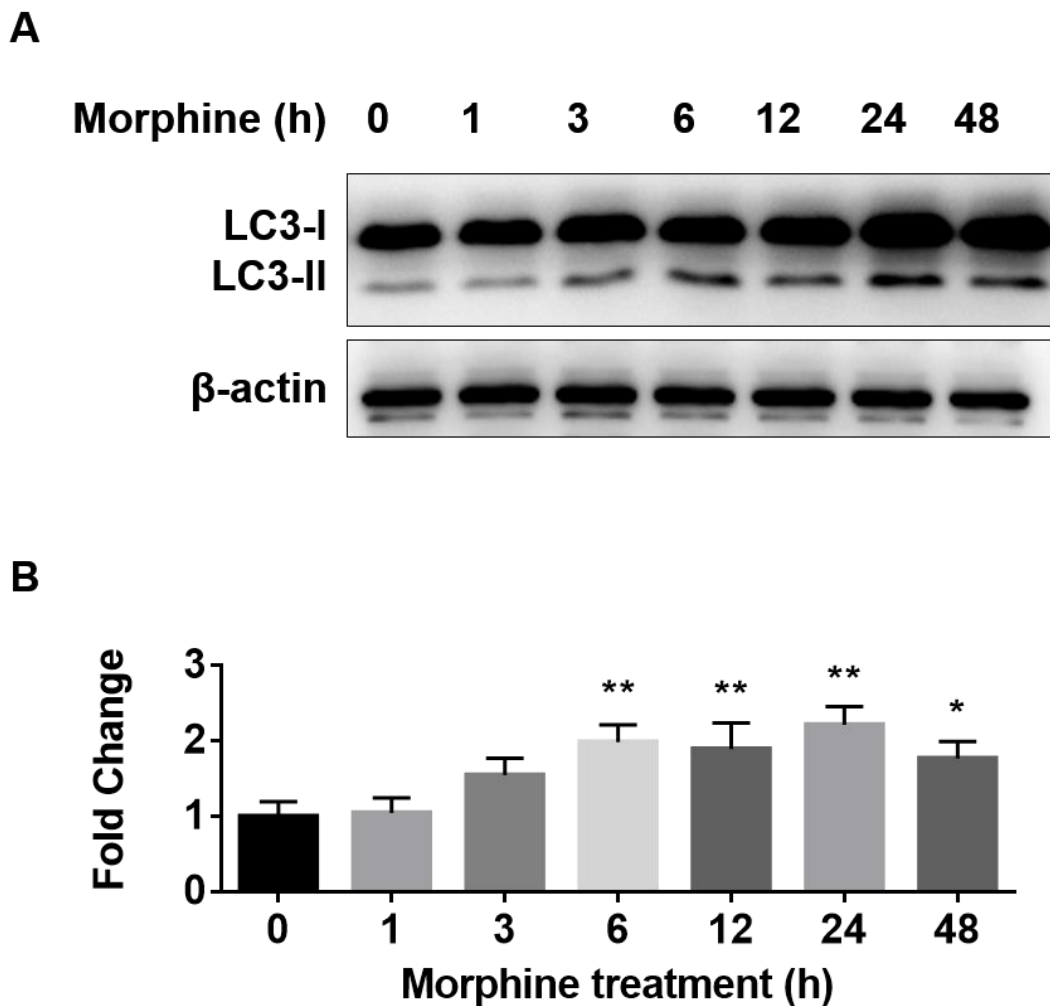
Figure 15.2 Morphine induces upregulation of LC3-II in a time-dependent manner in hippocampal neurons.

(A) Representative time course western blot and (B) quantification of LC3-II in primary neurons exposed to morphine. Quantification of western blot results were all normalized to β-actin. Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, * P<0.05, ** P<0.01 vs control group using one-Way ANOVA analysis.

A

B
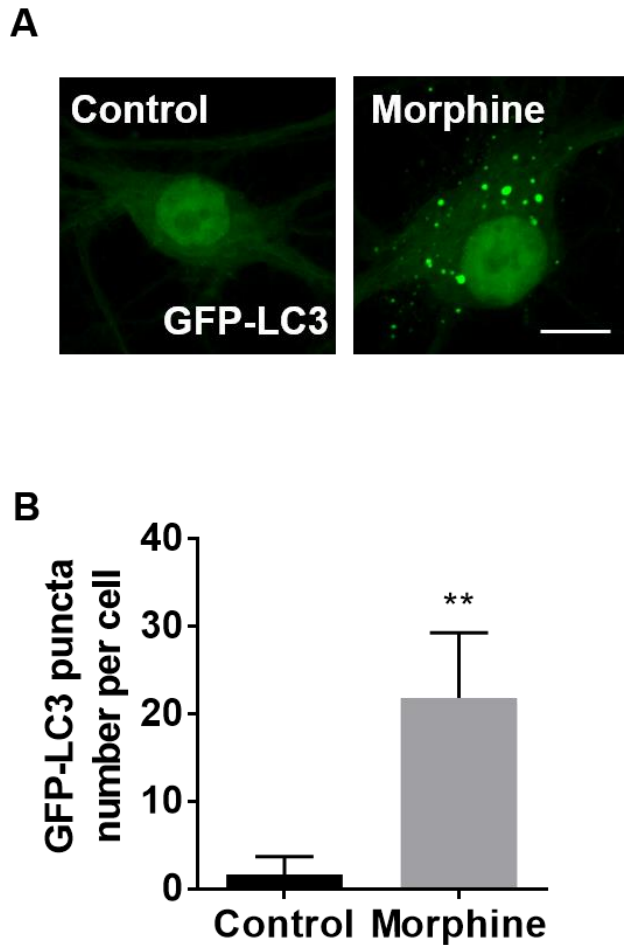
Figure 15.3 Morphine induces upregulation of GFP-LC3 puncta in hippocampal neurons.

(A) Representative confocal images and (B) quantification of GFP-LC3 puncta of primary hippocampal neurons expressing GFP-LC3 exposed to morphine for 24 h (scale bar = 10 μm). Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, ** $P<0.01$ vs control group using student's t test.

Figure 15.4 Morphine induces autophagy in hippocampal neurons *in vitro*.

(A) Representative western blot and (B) quantification of Beclin1, ATG5, p62 and LC3-II in the cell lysates from neurons pretreated with naltrexone for 1 h followed by 24-hour morphine treatment. Quantification of western blot results were all normalized to β-actin. Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control group; # $P<0.05$, ## $P<0.01$ vs morphine group using one-Way ANOVA analysis.

To determine whether morphine mediated enhanced generation of autophagosomes, morphine exposed primary rat neurons were treated with bafilomycin, an autophagy inhibitor (at a saturating concentration of 100 nM), to prevent fusion of autophagosomes with lysosomes (Shehata, Matsumura et al. 2012), and assessed for expression of LC3-II. As shown in Figures **15.5A & 15.5B**, there was a significant increase in LC3-II levels in neurons exposed to both morphine and bafilomycin compared to bafilomycin alone, indicating thereby that morphine induced autophagy via stimulating the formation of autophagosomes.

A

| Morphine | - | + | + | - |
| Bafilomycin | - | - | + | + |

LC3-I
LC3-II

β-actin

B
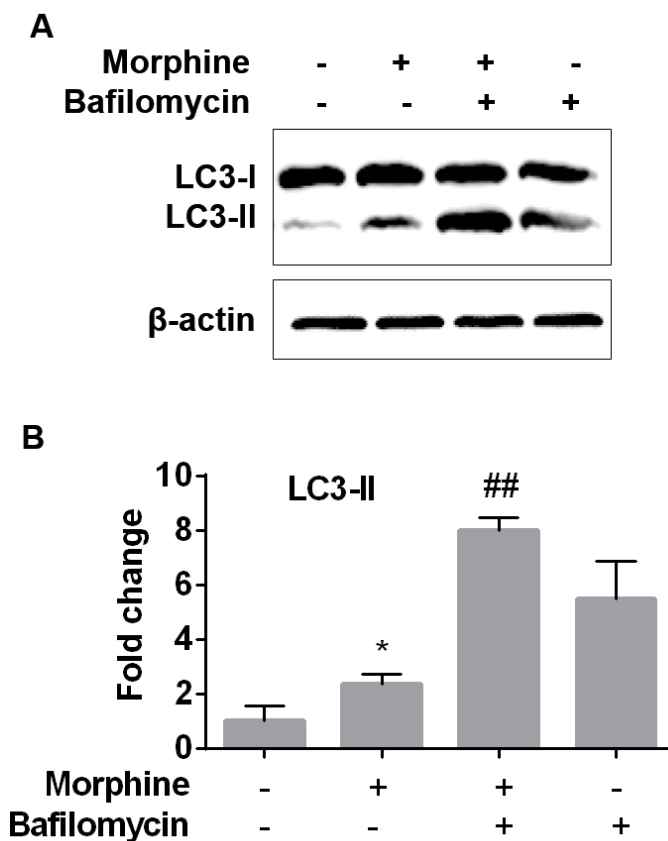
Figure 15.5 Morphine induces autophagosome formation in hippocampal neurons.

(A) Representative western blot and (B) quantification of LC3-II in the cell lysates of neurons treated with bafilomycin for 4 h following 24-hour exposure to morphine. Quantification of western blot results were all normalized to β-actin. Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, * P<0.05 vs control group; ## P<0.01 vs morphine group using one-Way ANOVA analysis.

16 The role of autophagy in morphine-mediated synaptic alterations

To explore the role of autophagy in morphine-mediated synaptic alterations, GFP-transfected neurons were pretreated with an autophagy inhibitor wortmannin that suppresses formation of autophagosomes, prior to exposure of cells to morphine. Pharmacological blocking of autophagy ameliorated morphine-mediated loss of spine density and excitatory synapses with an increase in inhibitory synapses **(Figures 16.1A & 16.1B).**

To confirm that these results were specific for the autophagy pathway, we examined the effects of knocking down ATG7 (Rodriguez-Muela, Koga et al. 2013) on morphine-mediated synaptic alterations. Primary hippocampal neurons were transfected with either vector or shRNA to ATG7, followed by morphine exposure and assessed for densities of both excitatory and inhibitory synapses. Consistent with the data using chemical inhibitors of autophagy, knocking down ATG7 in neurons, alleviated the synaptic phenotype observed under the influence of morphine **(Figures 16.2A & 16.2B).** Taken together, these results indicate that autophagy is a critical regulator of morphine-mediated synaptic alterations.
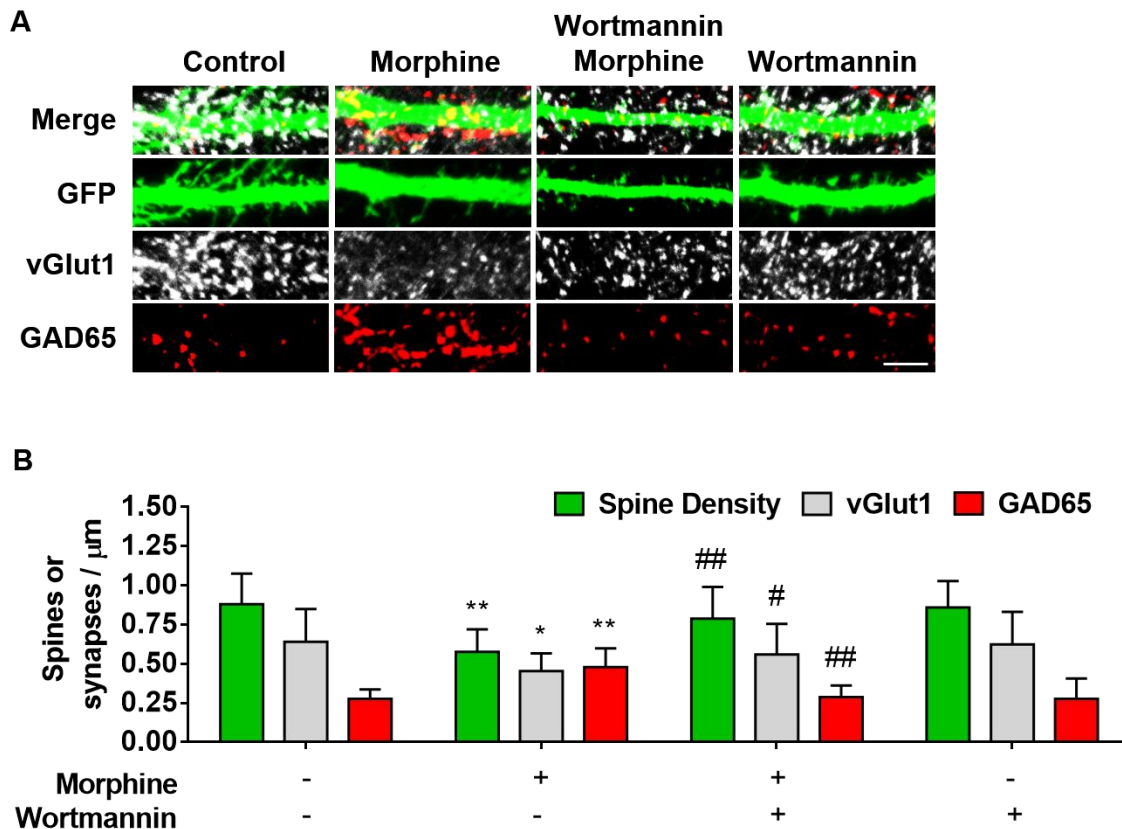
Figure 16.1 Autophagy inhibitor diminishes morphine-mediated synaptic alterations in hippocampal neurons.

(A) Representative confocal images of GFP-transfected primary rat hippocampal neurons treated with wortmannin (500 µM) for 1 h followed by morphine exposure for 24 h and stained with vGlut1 and GAD65 and (B) quantifications of spine density, excitatory and inhibitory synapses (scale bar = 5 µm). Each set of *in vitro* results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control group; # $P<0.05$, ## $P<0.01$ vs morphine group using one-Way ANOVA analysis.
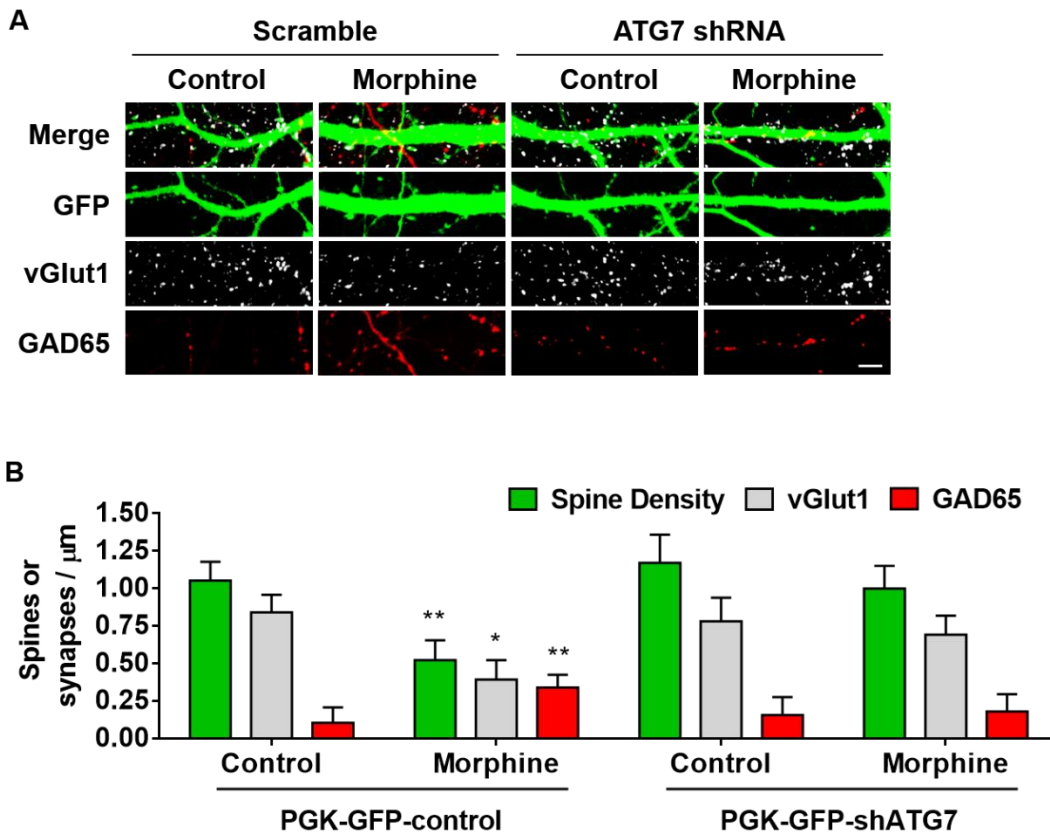
Figure 16.2 ATG7 shRNA diminishes morphine-mediated synaptic alterations in hippocampal neurons.

(M) Representative confocal images of neurons expressing PGK-GFP-shControl or PGK-GFP-shATG7 vector in the presence or absence of morphine for 24 h and stained with vGlut1 and GAD65 and (N) quantifications of spine density, excitatory and inhibitory synapses (scale bar = 5 µm). Quantification of western blot results were all normalized to β-actin. Each set of *in vitro* results were quantified upon four independent experiments. All data are 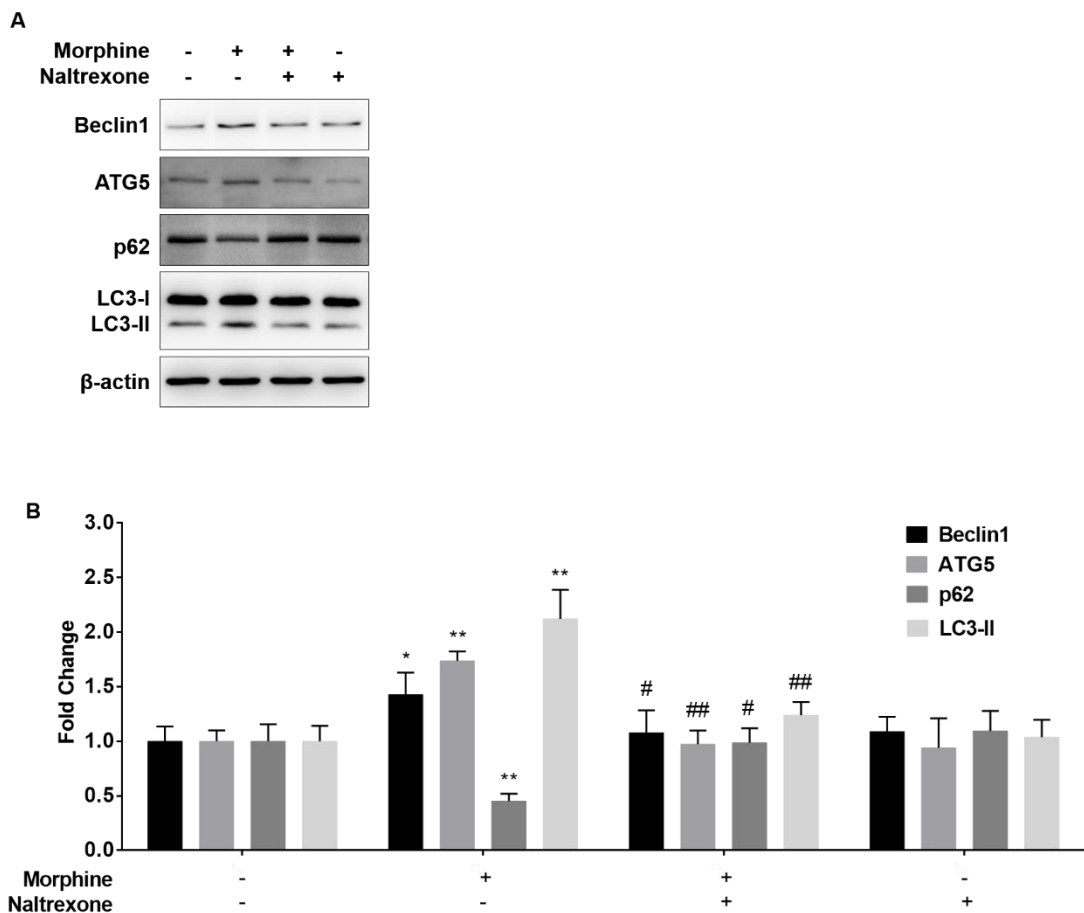presented as mean ± SD, * P<0.05, ** P<0.01 vs control group; # P<0.05, ## P<0.01 vs morphine group using one-Way ANOVA analysis.

17 The role of oxidative and ER stress in morphine-mediated autophagy

Having determined the involvement of oxidative stress, ER stress and autophagy in morphine-mediated synaptic alterations, we next sought to examine the link between morphine -mediated oxidative & ER stress and autophagy. For this primary hippocampal neurons were pretreated with various pharmacological inhibitors specific for oxidative or ER stress followed by exposure of cells to morphine and assessment of autophagic markers by western blotting. As shown in **Figures 17.1A & 17.1B**, in the presence of the oxidative stress inhibitors PBN or apocynin, morphine failed to upregulate the expression of major autophagy mediators such as Beclin1, ATG5 and LC3-II and also failed to downregulate the expression of autophagy substrate p62. Interestingly, autophagy inhibitor 3-MA failed to have any effect on morphine-mediated ROS production **(Figure 17.2)**. Similarly, in the presence of ER stress inhibitors such as 4-PBA or salubrinal, morphine failed to regulate autophagy mediators **(Figures17.3A & 17.3B)**. Reciprocally however, pretreatment of cells with autophagy inhibitors such as wortmannin or 3-MA had no effect on morphine-mediated upregulation of ER stress proteins **(Figures 17.4A & 17.4B)**. Taken together, these findings indicate that both oxidative and ER stress are upstream of morphine-mediated autophagy.

A

Morphine	-	+	+	+	-	-
PBN	-	-	+	-	+	-
Apocynin	-	-	-	+	-	+

Beclin1

ATG5

p62

LC3-I
LC3-II

β-actin

B
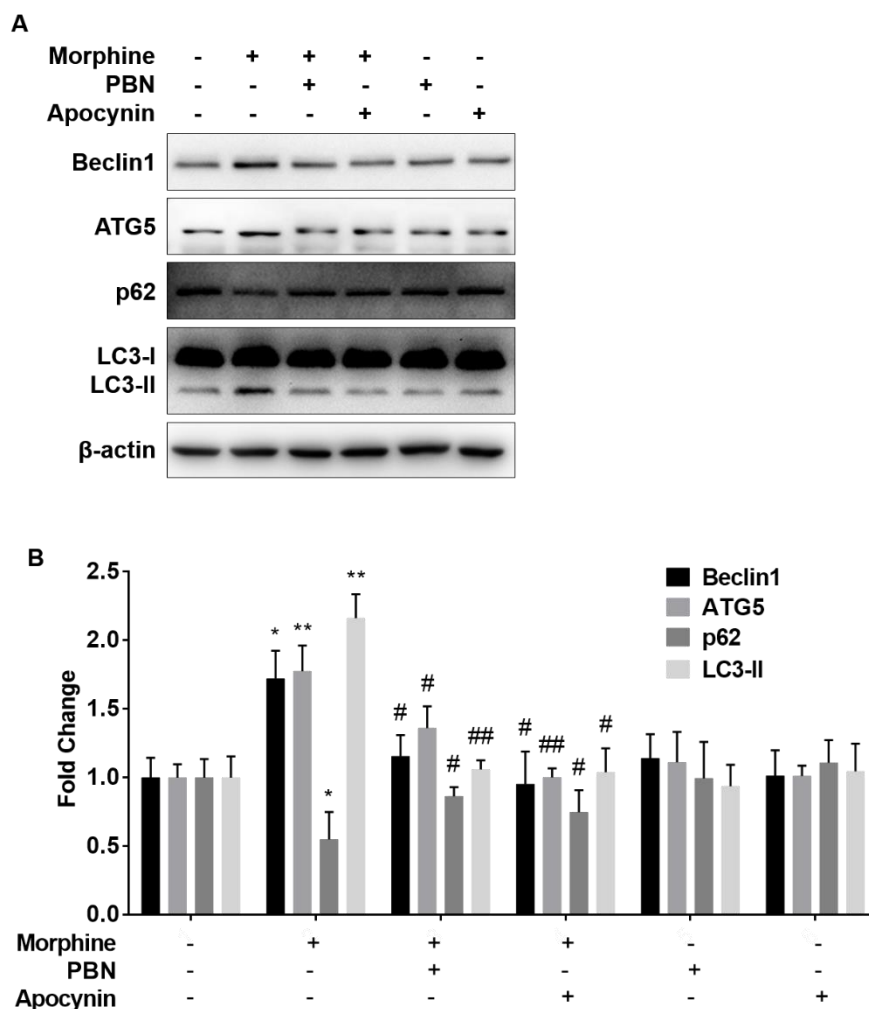
Figure 17.1 Oxidative stress plays a role in morphine-mediated autophagy in hippocampal neurons.

(A) Representative western blot and (B) quantification of autophagy mediators (Beclin1, ATG5, p62 and LC3-II) in the cell lysates of neurons pretreated with PBN or apocynin for 1 h followed by 24-hour morphine treatment. Quantification of western blot results were all normalized to β-actin. Each set of our results were quantified upon four independent experiments. All data are presented as mean ± SD, * P<0.05, ** P<0.01 vs control/saline group; # P<0.05, ## P<0.01 vs morphine group using one-Way ANOVA analysis.
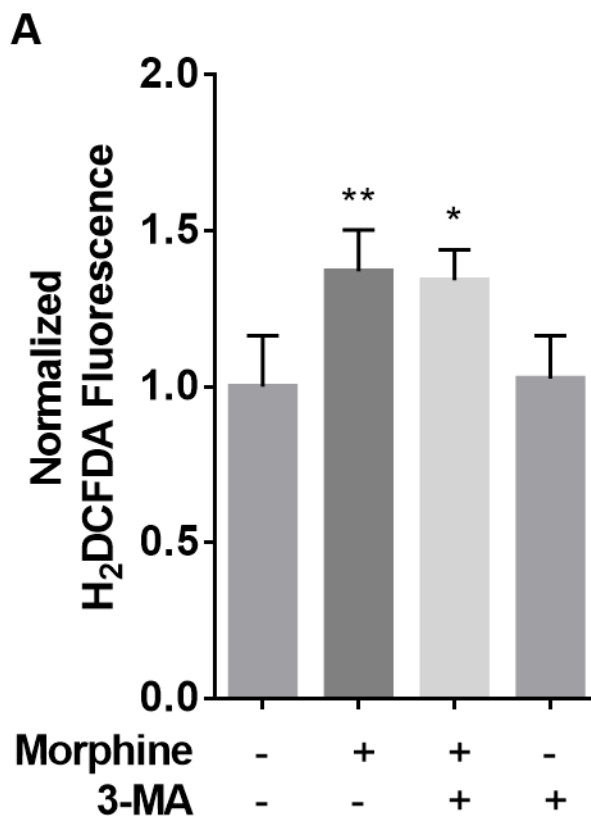
Figure 17.2 Inhibition of autophagy does not suppress morphine-mediated oxidative stress in hippocampal neurons.

(A) H_2DCFDA assay of neurons pretreated with 3-MA (50 µM) for half an hour followed by morphine exposure. Quantification of western blot results were all normalized to β-actin except that pPERK was normalized to PERK. Each set of our results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control/saline group; # $P<0.05$, ## $P<0.01$ vs morphine group using one-Way ANOVA analysis.

A

Morphine	-	+	+	+	-	-
4-PBA	-	-	+	-	+	-
Salubrinal	-	-	-	+	-	+

Beclin1

ATG5

p62

LC3-I
LC3-II

β-actin

B
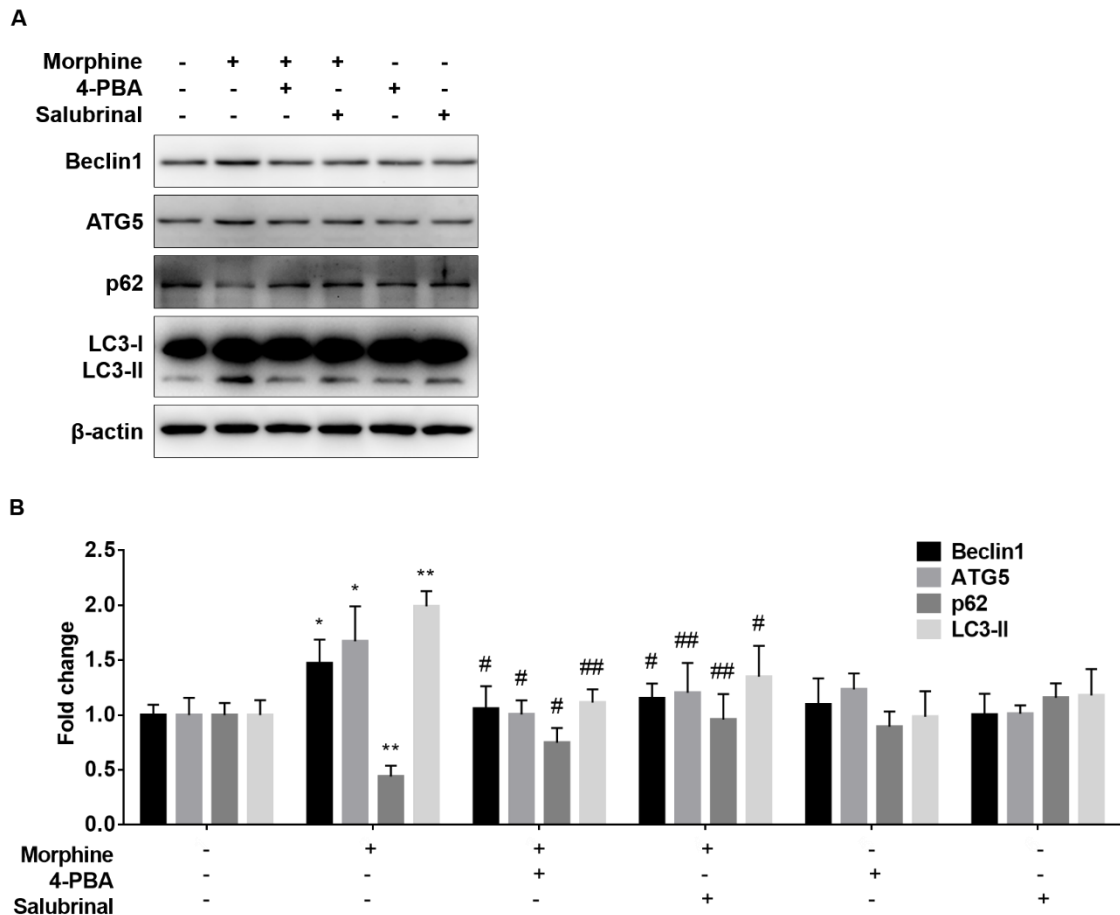
Figure 17.3 ER stress plays a role in morphine-mediated autophagy in hippocampal neurons.

(A) Representative western blot and (B) quantification of Beclin1, ATG5, p62 and LC3-II in the cell lysates of neurons pretreated with 4-PBA or salubrinal (100 μM) for 1 h followed by 24-hour morphine exposure. Quantification of western blot results were all normalized to β-actin. Each set of our results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control/saline group; # $P<0.05$, ## $P<0.01$ vs morphine group using one-Way ANOVA analysis.

Figure 17.4 Inhibition of autophagy does not suppress morphine-mediated ER stress in hippocampal neurons.

(A) Representative western blot and (B) quantification of BIP, pPERK, IRE1α and ATF6 in the cell lysates of neurons pretreated with wortmannin or 3-MA for 1 h followed by 24-hour morphine exposure. Quantification of western blot results were all normalized to β-actin except that pPERK was normalized to PERK. Each set of our results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control/saline group; # $P<0.05$, ## $P<0.01$ vs morphine group using one-Way ANOVA analysis.

18 Discussion

Neural circuits are regulated by activity dependent feedback that tightly control neural excitability. Deficits or imbalances in excitation and inhibition can have serious consequences that contribute to pathological conditions. Such perturbations are frequently observed in neurodevelopmental disorders associated with cognitive disabilities (Sandi and Haller 2015). Thus, it is likely that morphine mediated alterations in the balance of excitatory and inhibitory synapses contribute to deficits in the neural circuits underlying cognition.

Autophagy is an intracellular process that delivers cellular organelles and misfolded proteins from the autophagosomes to the lysosomes for degradation (Zhang and Baehrecke 2015). Substances of abuse such as cocaine and morphine have been shown to induce autophagy in the human neuroblastoma cell line SH-SY5Y and rat hippocampus (Zhao, Zhu et al. 2010) and microglia (Guo, Liao et al. 2015).

Our understanding of the link between autophagy and synaptic structure and function in absence of neurodegeneration (Banerjee, Beal et al. 2010, Wong and Cuervo 2010, Menzies, Fleming et al. 2015, Martini-Stoica, Xu et al. 2016), has been limited. Recent studies have implicated a role for autophagy in spine pruning associated with autism (Tang, Gudsnuk et al. 2014) via an mTORC1 dependent pathway. However, there are no reported studies that link autophagy to the morphogenesis of inhibitory synapses.

Our studies indicate that morphine-mediated induction of autophagy regulates both excitatory and inhibitory synapses. Moreover, we demonstrate that morphine initiates the autophagy cascade via a sequential activation of ROS and ER stress pathways and results in enhanced autophagosome formation. In addition to the implications of these

studies for morphine mediated synaptic changes, this is the first study to implicate a role for autophagy in the regulation of inhibitory synapses. How induction of autophagy leads to alterations in synaptic densities, particularly inhibitory synapses, remains to be identified. During the development of the hippocampus, excessive synapses are generated prior to active pruning to generate a fairly stable synaptic pool (Tang, Gudsnuk et al. 2014). It is conceivable that autophagic mechanisms operate either at the level or synapse generation or synaptic pruning to control synaptic densities.

CHAPTER VII

Protective role of PDGF-BB in morphine-mediated

synaptic alterations

19 PDGF-BB reverses morphine-mediated synaptic alterations

Our previous studies have demonstrated a neuroprotective role of PDGF-BB in both primary cortical neurons and human neuroblastoma cell line against HIV proteins Gp120 and Tat (Peng, Dhillon et al. 2008, Peng, Dhillon et al. 2008, Zhu, Yao et al. 2009). We have also previously demonstrated that exposure of hippocampal neurons to PDGF-BB leads to induction of the gene Arc/Arg3.1 (Peng, Yao et al. 2010), a synaptic plasticity gene that plays a critical role in learning and memory formation (Korb, Wilkinson et al. 2013, Cao, Ye et al. 2015, Gouty-Colomer, Hosseini et al. 2016). Moreover, PDGF-BB is also known to protect hippocampal neurons against apoptosis induced by glucose deprivation or an oxidative insult (Cheng and Mattson 1995). We thus rationalized that exposure of morphine-treated neurons to PDGF-BB could also lead to protection against morphine-mediated impairment of synaptic alterations via its effects on ROS and downstream ER stress and autophagy. To verify this, hippocampal primary neurons (DIV 20) were treated with PDGF-BB (20 ng/ml) for 24 hours, following exposure to morphine. After treatment, neurons were fixed, immunostained for synaptic markers and subjected to confocal microscopy to assess synaptic densities. In neurons treated with PDGF-BB, morphine exposure did not alter synaptic densities, unlike neurons exposed to morphine, suggesting thereby that PDGF-BB treatment resulted in significant protection against morphine-mediated alterations in synaptic densities **(Figures 19A & 19B).**
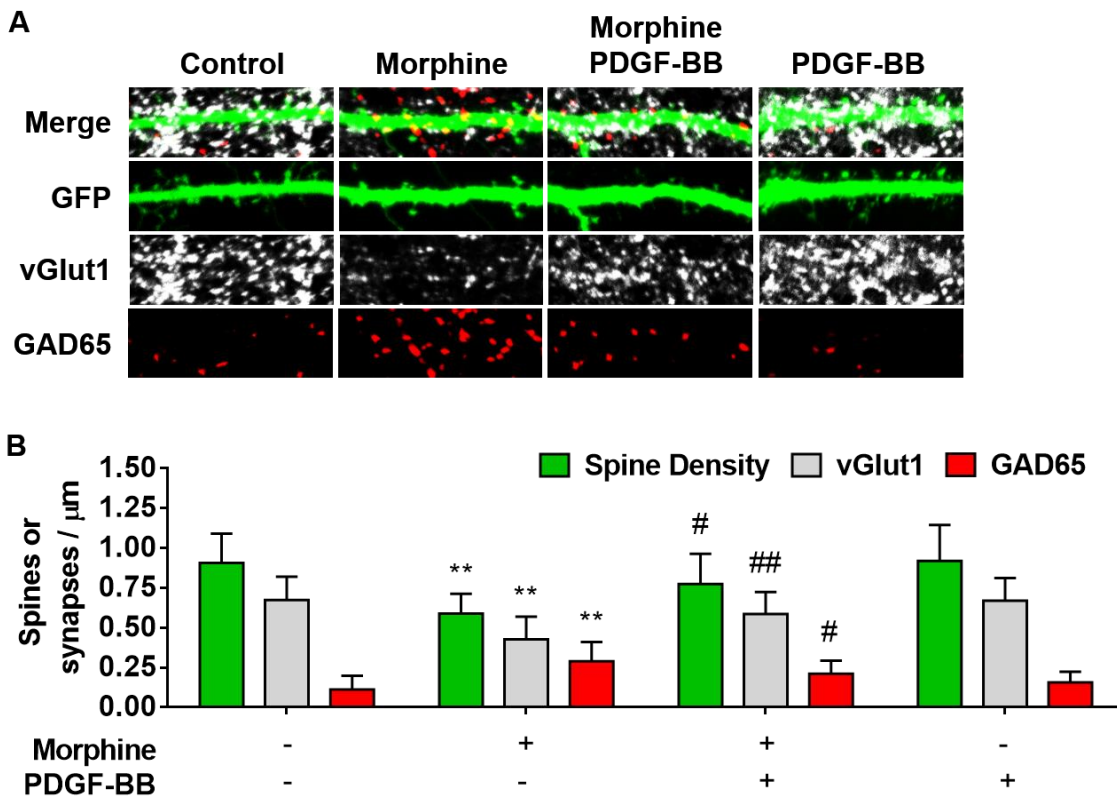
Figure 19 PDGF-BB reverses morphine-mediated synaptic alterations in hippocampal neurons.

(A) Representative confocal images of GFP-transfected primary rat hippocampal neurons exposed to morphine followed by treatment with PDGF-BB (20 ng/ml) and stained with vGlut1 and GAD65 and (B) quantifications of spine density, excitatory and inhibitory synapses (scale bar = 5 μm). Each set of our results were quantified upon four independent experiments. All data are presented as mean ± SD, ** P<0.01 vs control group; # P<0.05, ## P<0.01 vs morphine group using one-Way ANOVA analysis.

20 PDGF-BB prevents activation of ROS-ER stress-autophagy axis in neurons exposed to morphine

PDGF-BB exposure also resulted in amelioration of morphine-mediated ROS production **(Figure 20.1A)** as examined by H_2DCFDA fluorescence assay. Furthermore, western blot analyses also indicated that PDGF-BB treatment prevented the activation of ER stress **(Figures 20.2A & 20.2B)** & autophagy **(Figures 20.3A & 20.3B)** pathways, that lie downstream of morphine-mediated oxidative stress. Taken together, these results indicate that treatment of neurons with PDGF-BB is beneficial in ameliorating morphine-mediated synaptic alterations by inhibiting the generation of the upstream ROS that triggers activation of ER stress and autophagy pathways.
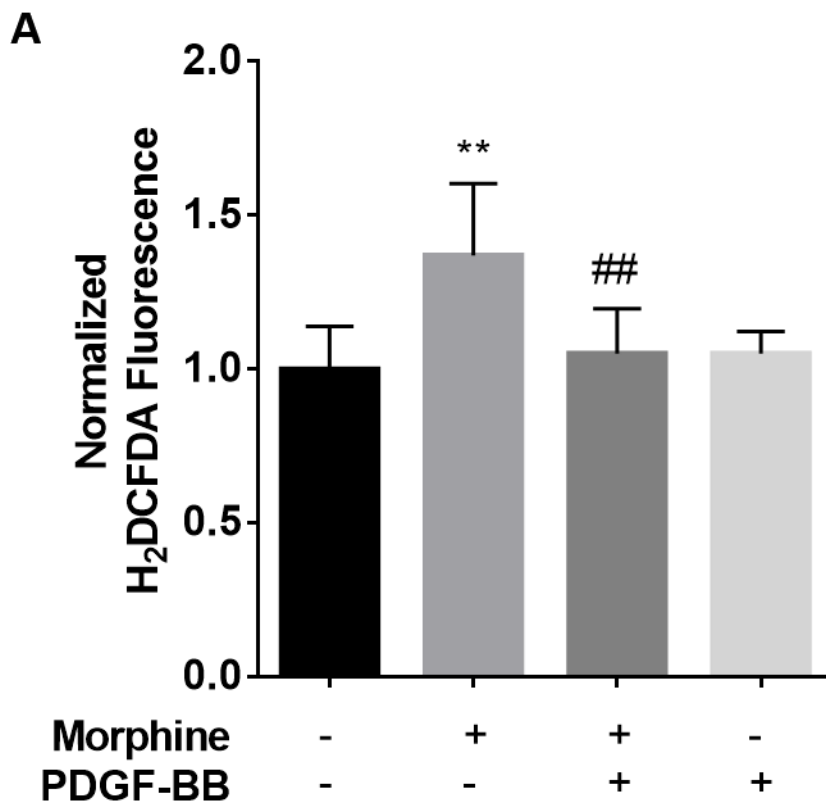
Figure 20.1 PDGF-BB reverses morphine-mediated oxidative stress in hippocampal neurons.

(A) ROS generation in neurons exposed to morphine and treated with PDGF-BB. Each set of our results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control/saline group; # $P<0.05$, ## $P<0.01$ vs morphine group using one-Way ANOVA analysis.
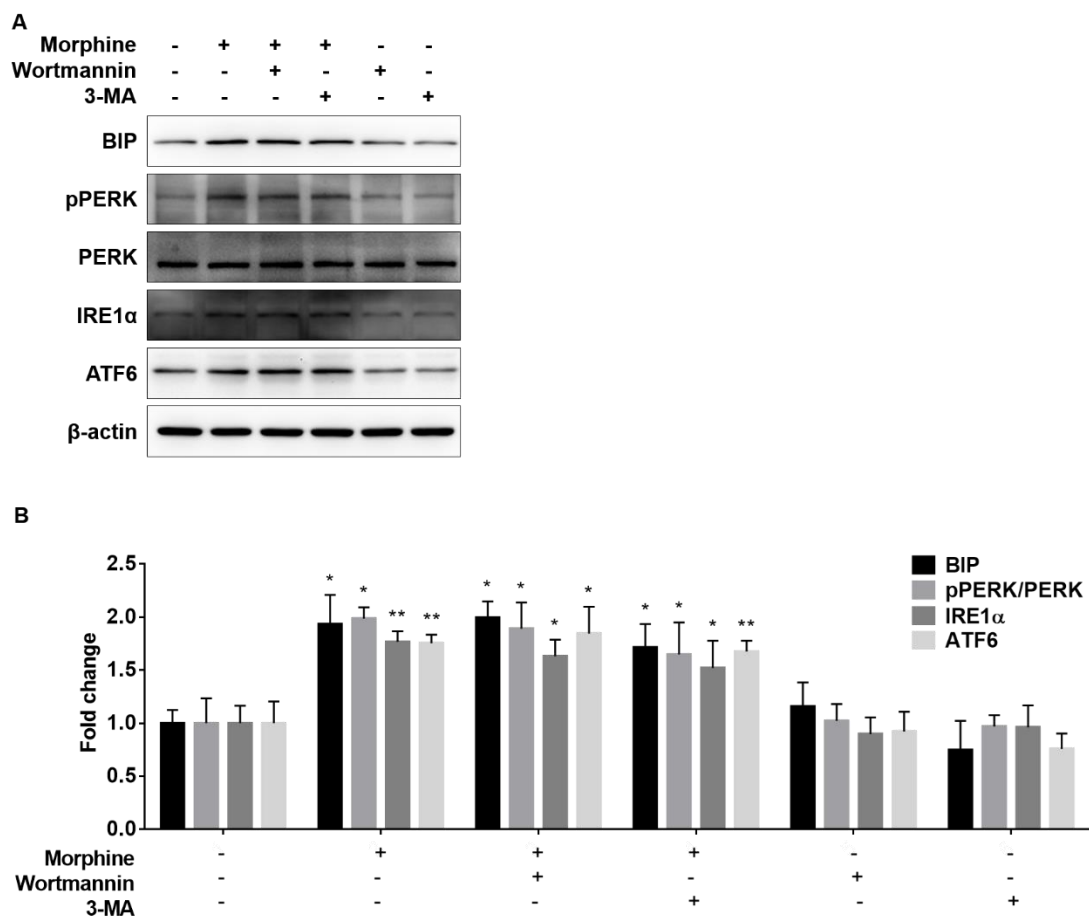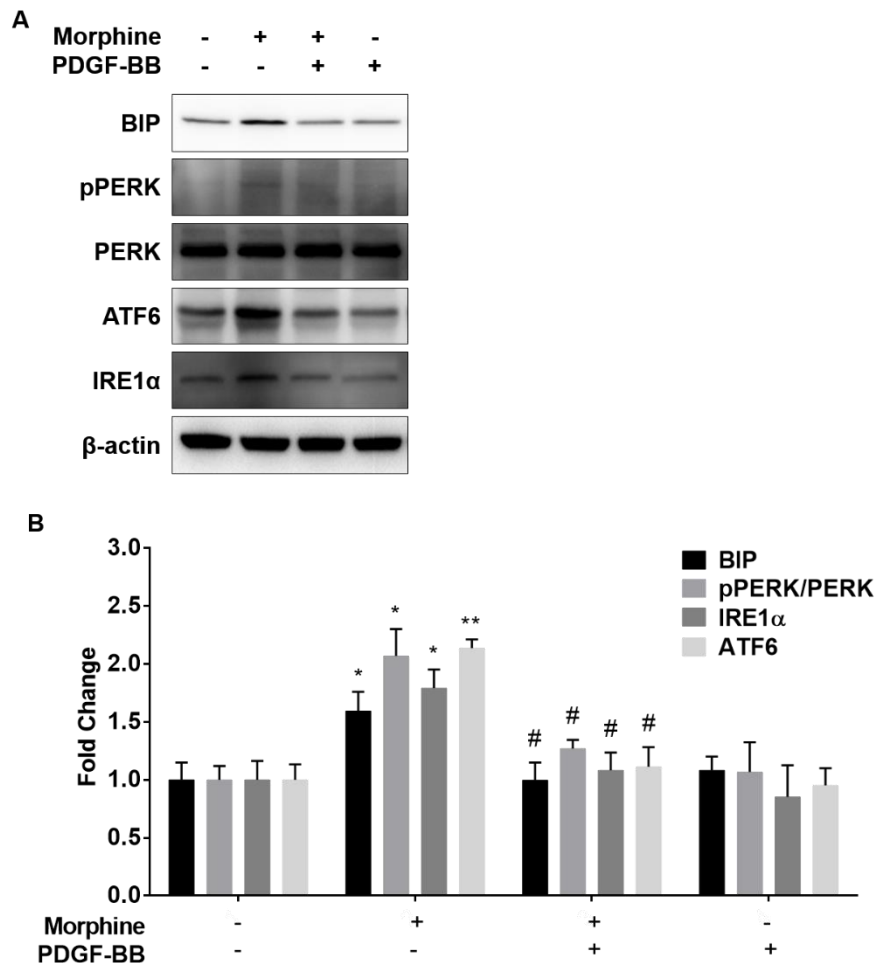
Figure 20.2 PDGF-BB reverses morphine-mediated ER stress in hippocampal neurons.

(A) Representative western blot and (B) quantification of BIP, pPERK, IRE1α and ATF6 in the cell lysates of neurons exposed to morphine followed by treatment with PDGF-BB (20 ng/ml) for additional 24 h. Quantification of western blot results were all normalized to β-actin except that pPERK was normalized to PERK. Each set of our results were quantified upon four independent experiments. All data are presented as mean ± SD, * P<0.05, ** P<0.01 vs control/saline group; # P<0.05, ## P<0.01 vs morphine group using one-Way ANOVA analysis.
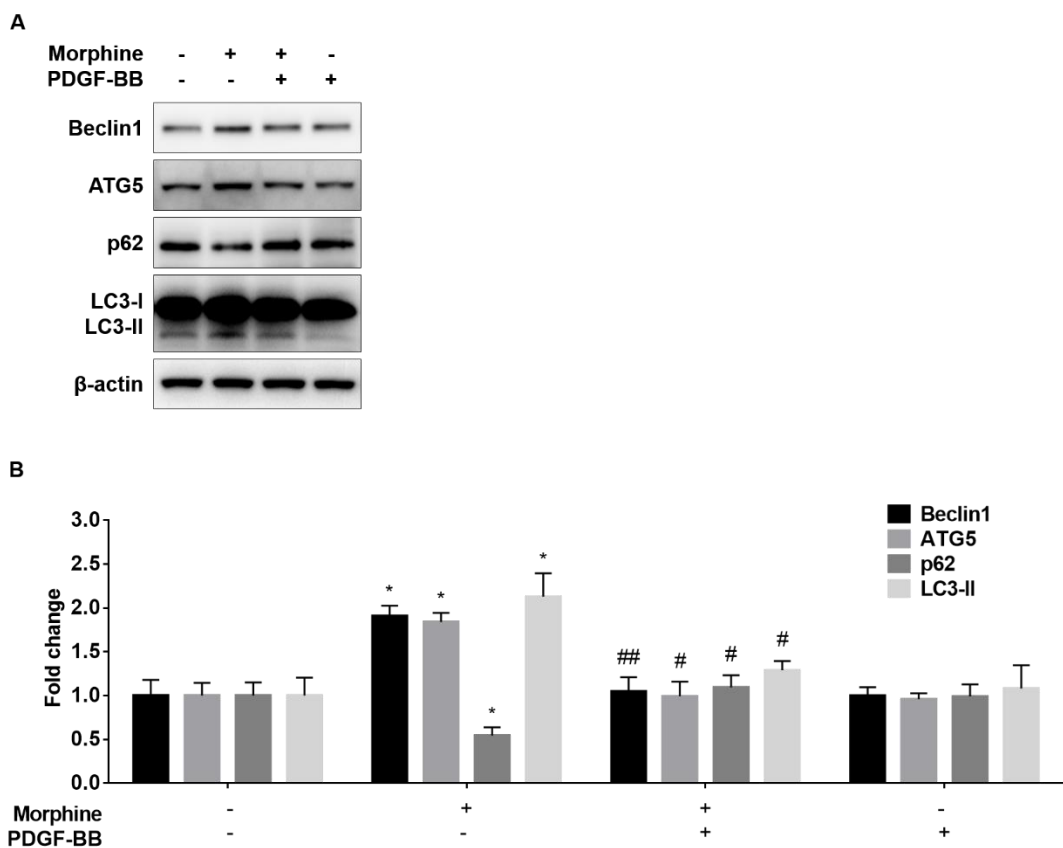
Figure 20.3 PDGF-BB reverses morphine-mediated autophagy in hippocampal neurons.

(A) Representative western blot and (B) quantification of Beclin1, ATG5, p62 and LC3-II in the cell lysates of neurons exposed to morphine for 24 h followed by PDGF-BB (20 ng/ml) for additional 24 h. Quantification of western blot results were all normalized to β-actin. Each set of our results were quantified upon four independent experiments. All data are presented as mean ± SD, * $P<0.05$, ** $P<0.01$ vs control/saline group; # $P<0.05$, ## $P<0.01$ vs morphine group using one-Way ANOVA analysis.

21 Discussion

PDGF-BB, a robust pleiotropic factor, is neuroprotective in HIV protein Gp120- and Tat-mediated apoptosis (Peng, Dhillon et al. 2008, Peng, Dhillon et al. 2008, Zhu, Yao et al. 2009) (Cheng and Mattson 1995) and has also been shown to restore neuronal differentiation in neural progenitor cells in the presence of both HIV Tat and cocaine (Yang, Chen et al. 2015). We (Peng, Yao et al. 2010) have previously demonstrated that PDGF-BB induces the expression of synaptic plasticity gene Arc/Arg3.1 in hippocampal neurons, suggesting that it may be a key player in the regulation of synaptic plasticity, although the underlying mechanisms remain unclear.

Our data indicates that treatment of morphine-exposed neurons with PDGF-BB ameliorated morphine-mediated synaptic alterations in hippocampal neurons. This effect of PDGF-BB was at the level of inhibition of ROS production following activation of MOR, leading in turn, to inhibition of downstream ER stress and autophagy pathways, ultimately leading to preservation of excitatory and inhibitory synapses.

CHAPTER VIII

The role of cocaine & Sigma-1 receptor in cocaine-related neurologic impairments

22 MULTIPLE-FACETED ROLES OF COCAINE IN POTENTIATION OF HAND

22.1 Overview

While the advent of HAART has successfully suppressed virus replication in infected individuals resulting in increased longevity of these patients, as well as, reduced the incidence of the more severe form of HIV-1-associated dementia (HAD) [4-7], the prevalence of milder forms of cognitive impairment collectively termed as HIV-associated neurocognitive disorders (HAND) remains high (Maschke, Kastrup et al. 2000, Gray, Chretien et al. 2003) (Maschke, Kastrup et al. 2000, Cherner, Masliah et al. 2002, Sacktor, McDermott et al. 2002). In fact almost 40-60% of infected individuals on HAART suffer from HAND and out of those almost 30% of have some form of substance abuse dependence. Furthermore, the limited ability of HAART to penetrate the blood-brain barrier (BBB) coupled with the early entry of HIV into the CNS within days of infection, establishes the brain as a virus sanctuary. However, interestingly, CNS viral loads do not correlate well with symptoms of HAND. In the current era of HAART, HIV replication is not the primary driving force for HAND pathogenesis, it is instead the residual expression of viral proteins such as HIV Tat and the envelope protein Gp120 that are found in significant levels in tissues and remain elevated even in the presence of HAART which are the likely cause of tissue toxicity and chronic low-level inflammation (Spudich and Gonzalez-Scarano 2012).

As the lifespan of infected patients continues to increase with HAART, signs of premature aging are becoming more prominent with an earlier onset of many age-related co-morbidities in these individuals (Hauser, Fitting et al. 2012). Toxicity related to HAART medications and chronic exposure to viral proteins are thought to play an influential role in accelerating the aging process (Smith, de Boer et al. 2012). Drugs of abuse also likely play a similar role as it has been shown that chronic drug abuse itself leads to accelerated

aging (Piggott, Muzaale et al. 2013). In fact, it is well recognized that substance abuse and HIV-1 infection are two closely linked global health crises. Although in recent years substance abuse among the population of HIV infected patients has remained relatively constant, it still accounts for a significant number of new infections (Teplin, Elkington et al. 2005). It is well acknowledged that intravenous drug use is a significant risk factor for acquiring HIV due to needle sharing and subsequent risky behaviors associated with its use. Furthermore, substance abuse in general, including cocaine abuse, may also play a significant role in the transmission and pathogenesis of HIV since it has been demonstrated to not only affect many facets of the immune system but also directly accelerate the rate of viral replication in many cell types (Buch, Yao et al. 2012). Taken together, the interaction of HIV, HAART, aging and drug of abuse all contribute to underlying inflammation, leading to the age-related comorbidities observed in infected patients. This review comprehensively summarizes the varied functional implications of cocaine in various CNS cell types and brings together their outcomes to better understand how each contributes to the progression and exacerbation of HAND.

The CNS is a major target for eliciting cocaine's psychostimulant effects. Cocaine is also able to impair the functions of macrophages and lymphocytes and enhance the replication of HIV in these cells (Klein, Matsui et al. 1993, Mao, Huang et al. 1996, Baldwin, Tashkin et al. 1997). Cocaine has been shown to modulate the function of the blood brain barrier (BBB) and facilitate leukocyte infiltration (Sharma, Muresanu et al. 2009, Yao, Kim et al. 2011, Kousik, Napier et al. 2012). It is widely thought that disruption of BBB integrity is the primary mechanism of HIV entry into the CNS, thus it has been postulated that cocaine may contribute to the susceptibility and progression of HAND. The neuroinflammatory responses present in HAND involve the influx of both HIV-infected and uninfected cells into the CNS, where they activate and/or infect the resident glial cells

ultimately culminating into a self-propelled cycle of disease pathogenesis (Gannon, Khan et al. 2011). Cocaine has also been implicated to directly induce up-regulation of pro-inflammatory mediators such as cytokines and chemokines leading to neuroinflammation and transmission of toxic responses to resident CNS cells including neurons (Fox, D'Sa et al. 2012, Clark, Wiley et al. 2013), thus serving as a co-factor in the progression of HIV-1-associated neuropathogenesis **(Figure 22)**.
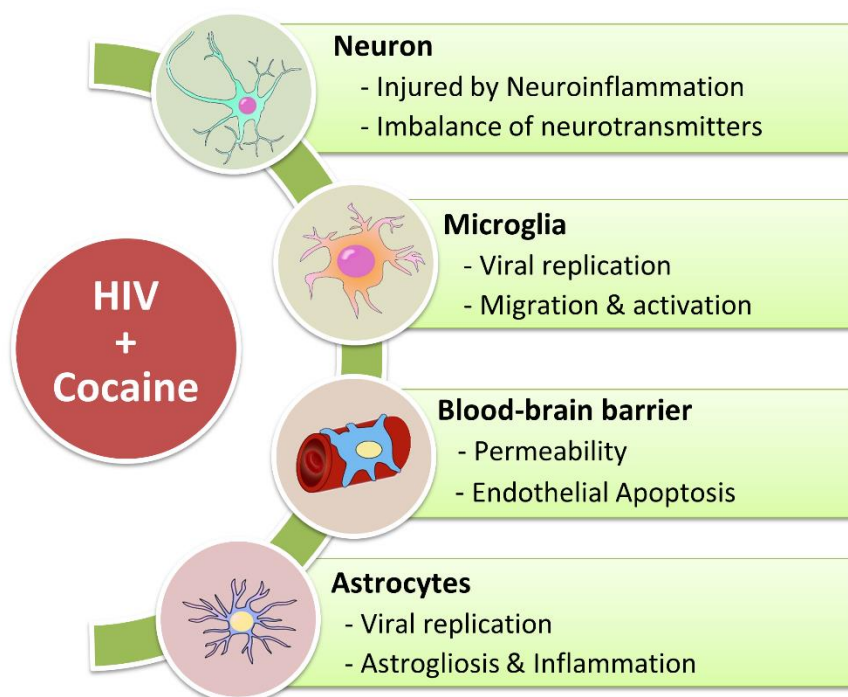
Figure 22 Schematic diagram depicting the interplay of HIV and cocaine.

In the presence of HAND, cocaine enhances viral replication in both microglia and astrocytes, increases permeability and apoptosis of endothelial cells, stimulates neuroinflammation and leads to neuronal injury (image used with permission from (Cai, Yang et al. 2016)).

22.2　Blood brain barrier (BBB) and cocaine

The blood-brain barrier (BBB) is a diffusion barrier composed a several cell types situated between the blood and brain parenchyma which has an important role in maintaining a highly regulated microenvironment to protect the CNS from infectious agents and toxins in the circulating system(Ballabh, Braun et al. 2004). Compromised BBB integrity plays a major role in the progression of HIV-1 infection as well as the manifestation of HAND in the era of HAART (Dallasta, Pisarov et al. 1999). BBB integrity is disrupted throughout the pathogenesis of neuroAIDS by various mechanisms including a reduction in BBB tight junction protein expression and loss of associated cells that leads to increased transmigration of HIV-infected monocytes (Eugenin, Osiecki et al. 2006). Cocaine use is also known to enhance the viral neuroinvasion by altering blood brain barrier integrity via distinct intracellular mechanisms (Fiala, Gan et al. 1998, Gan, Zhang et al. 1999, Chang, Bersig et al. 2000, Lee, Hennig et al. 2001). Cocaine is able to "open" the BBB through both direct pro-apoptotic effects on the endothelial cells as well as indirect paracrine effects that are manifested by pro-inflammatory modulators such as chemokines and cytokines (Zhang, Looney et al. 1998). Exposure of cocaine to human brain endothelial cells has been shown to decrease cellular glutathione levels, enhance DNA-binding activity of redox-regulated transcription factors such as NF-kB and AP-1 and increase expression of pro-inflammatory cytokine TNF-α, thereby contributing to BBB dysfunction and enhanced leukocyte migration across cerebral vessels (Lee, Hennig et al. 2001). Cocaine has been shown to increase the expression of several endothelial adhesion molecules in brain endothelial cells, such as intracellluar adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin. Up-regulation of these molecules contributes to increased leukocyte transmigration across the endothelial monolayers (Gan, Zhang et al. 1998, Gan, Zhang et al. 1999). Previous studies conducted by our group

demonstrated that HIV+ cocaine abusers express higher levels of activated leukocyte cell adhesion molecule (ALCAM) in the brain endothelium accompanied by increased monocyte/macrophage infiltration when compared with HIV+ individuals with no drug abuse history or uninfected controls. Similarly, a report by Chang et al. indicated that chronic cocaine treatment is able to potentiate chemokine-induced leukocyte-endothelial cell adhesion (LEA) in rats (Chang, Bersig et al. 2000). In addition to potentiation of cell adhesion molecules, our previous study also indicated that cocaine enhances the permeability of brain endothelial cells through upregulation of PDGF-BB which acts as a vascular permeant (Yao, Duan et al. 2011). The underlying molecular mechanisms involved in this process include activation of MAPKs and the transcription factor Egr-1. This was further confirmed in Egr-1 knockout mice wherein cocaine failed to enhance endothelial permeability. Furthermore, cocaine has also been shown to increase barrier permeability and endothelial transmigration of HIV-infected dendritic cells via up-regulation of dendritic cell-specific C type ICAM-3 grabbing non-integrin (DC-SIGN) and matrix metalloproteinases in HBMECs (Nair, Mahajan et al. 2000).Finally, a recent study reported that cocaine can remodel HBMECs by up-regulating transcription of genes critical for cytoskeletal organization, signal transduction, cell swelling and vesicular trafficking (Fiala, Gan et al. 1998).

22.3   Effect of HIV and cocaine in mediating astrocyte dysfunction

Astrocytes, the most abundant cell type within the brain, have many important roles in the CNS including providing structural support, maintaining ion homeostasis, regulating synaptic communication, providing metabolic support and actively participating in communication with other CNS cells (Li, Zhao et al. 2011). Although astrocytes are not a primary source of HIV replication in the CNS, they provide an important reservoir for the

generation of inflammatory mediators in response to either HIV or its associated viral toxins(Conant, Tornatore et al. 1994, Brack-Werner 1999, Canki, Thai et al. 2001).Once activated, astrocytes undergo astrogliosis characterized by increased expression of intermediate filament proteins, in particular glial fibrillary acidic protein (GFAP), as well as the release of several different cytokines and chemokines(Li, Zhao et al. 2011). Cocaine, a known cofactor in HIV pathogenesis, contributes to increased HIV infectivity of CNS astrocytes. For example, it has been reported that exposure of astrocytes to cocaine prior to HIV infection significantly increases viral replication as evidenced by LTR-R/U5 gene expression, an indication of increased reverse transcription of HIV-1 virus (Reynolds, Mahajan et al. 2006). In addition to enhancing virus replication in astrocytes, cocaine can also increase the expression of GFAP as confirmed in our recent studies thereby contributing to the progression of astrocyte activation(Yang, Yao et al. 2015). In this study, we found increased astrogliosis in brain sections from patients with both HIV and a history of cocaine abuse compared with either an HIV-infected non-abusing group or control group. Furthermore, we determined the molecular mechanism involved in cocaine-mediated astrogliosis whereby ligation of σ-1 receptor following cocaine exposure triggers its translocation to the plasma membrane leading to activation of the MAPK pathway cascade and subsequent downstream activation of the transcription factor Egr-1, which in turn, transcriptionally upregulates GFAP expression (Yang, Yao et al. 2015). Our study is consistent with a previous in vivo study revealing that cocaine administration to mice resulted in increased GFAP expression in the dentate gyrus. While prominent evidence indicates the role of cocaine in promoting astrocyte activation, it is also becoming clear that dampened astrocyte viability is also critical for the progression of HAND and can be potentiated by cocaine addiction in the context of HIV infection. A previous study from our group demonstrated that cocaine enhanced HIV viral envelope protein Gp120-mediated decrease in astrocyte viability. The detailed molecular mechanisms underlying this

phenomenon include cocaine-mediated generation of intracellular reactive oxygen species (ROS) and resulting imbalance in mitochondrial membrane potential which leads to activation of downstream signaling pathways involving MAPK and nuclear factor NF-Kb(Yang, Yao et al. 2010).

22.4   Inflammatory responses in microglia mediated by cocaine and HIV

The migration and activation of microglia/macrophages also plays a crucial role in modulating the trajectory of neurological disorders arising over the course HIV infection. Our previous studies have already solidified the correlation between the number of activated microglia/macrophages and neuroinflammation using macaque models of HAND (Smith, Niu et al. 2005). In addition to its ability to stimulate the reward system, cocaine is also capable of exacerbating neuroinflammatory responses in the brain via chemokines such as monocyte chemoattractant protein-1 (MCP-1) and signaling pathways including those regulating ER stress and autophagy (Yao, Yang et al. 2010, Costa, Yao et al. 2013, Guo, Liao et al. 2015). It has been well documented that marked upregulation of MCP-1 is observed in the brains of macaques infected with simian human immunodeficiency virus (SHIV) (Sui, Potula et al. 2003). Cocaine has also been reported to translocate its cognate receptor σ-1 to the lipid raft microdomains of the plasma membrane followed by sequential activation of Src, mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K)/Akt pathway and nuclear factor-κB (NF-κB), resulting in the enhanced expression of MCP-1 and recruitment of peripheral monocyte-derived macrophages across the BBB into the brain (Yao, Yang et al. 2010). Additionally, cocaine also mediates upregulation of activated leukocyte cell adhesion molecule (ALCAM) through translocation of σ receptor in a similar manner, resulting in the transmigration of peripheral monocyte across BBB (Yao, Kim et al. 2011). Interestingly,

HIV Tat protein has also been shown to mediate microglial activation and migration (Yao, Duan et al. 2013).Our recent study has identified that a unique role of microRNA-9 (miR-9), known for controlling neurogenesis, was upregulated by Tat and which could directly activate microglia and induce microglial inflammatory response via distinct signaling pathways. MiR-9-mediated regulation of cellular activation involved down regulated expression of the target protein, monocyte chemotactic protein-induced protein-1(MCPIP1) that is crucial for controlling inflammation (Yao, Ma et al. 2014). Earlier study also demonstrated that the interaction of Tat and vascular endothelial growth factor receptor type 1 (VEGF-R1) evokes activation of myosin light-chain kinase (MYLK) and β1 integrin in a temporal manner followed by actin polymerization, leading to microglial migration (Yao, Duan et al. 2013). In addition to microglial migration, evidence also suggests that both Tat and cocaine activate NF-κB, leading to microglial activation and the release of inflammatory factors (Dhillon, Williams et al. 2007, Duan, Yao et al. 2014). Intriguingly, a recent study has also demonstrated that two major ER stress pathways including PRKR-like endoplasmic reticulum kinase (PERK) and inositol-requiring enzyme 1 (IRE1) are involved in cocaine-mediated autophagy that ultimately culminates into the release of inflammatory factors from activated microglia (Guo, Liao et al. 2015).

22.5   Toxicity of cocaine and HIV viral proteins in neurons and neural progenitor cells (NPC)

HIV-1 virus, albeit unable to productively infect neurons, is still capable of inducing neuronal injury by either mediating neuroinflammation via activation of glial cells or directly eliciting neuronal damage prompted by viral proteins such as Tat and Gp120 (Sui, Potula et al. 2004, Smith, Niu et al. 2005, Peng, Yao et al. 2012). Gp120-mediated upregulation of CXCL10 in neurons invokes elevation of intracellular calcium in cultured neurons

leading to the activation of intrinsic apoptotic pathways and ultimately neuronal cell death (Sui, Potula et al. 2004, Sui, Stehno-Bittel et al. 2006) which can be reversed by platelet-derived growth factor-BB (PDGF-BB) (Peng, Dhillon et al. 2008). Similarly, disruption of calcium homeostasis has also been reported to play a critical role in tat-mediated neurotoxicity (Zhu, Yao et al. 2009). Mechanistic studies have further demonstrated that Tat downregulates the expression of endogenous PDGF-CC, conversely, exogenously applied PDGF-CC protects neurons against tat-mediated neurotoxicity by stimulating the PI3K/Akt pathway which requires elevation of [Ca2+] transient via activated transient receptor potential canonical (TRPC) channels (Peng, Yao et al. 2012). Besides PDGF-CC, PDGF-BB has also been indicated to play a neuroprotective role against Tat by activating two parallel but distinct pathways known as the extracellular signal-regulated protein kinase/calcium/cAMP-response element-binding protein (ERK/CREB) pathway and the PI3K/Akt pathway (Zhu, Yao et al. 2009). Intriguingly, [Ca2+] influx through activated TRPC channels is indispensable for PDGF-CC-mediated activation of the ERK/CREB pathway but not the PI3k/Akt pathway (Zhu, Yao et al. 2009). Mounting evidence also indicates that HIV infection can lead not only to neuronal dysfunction or loss, but can also negatively impact neurogenesis, resulting in generation of fewer adult neural progenitor cells (NPCs) in the dentate gyrus of the hippocampus, brain area critical for memory and learning. The crucial role of PDGF-BB in providing tropic support for the neurons as well as in promoting neurogenesis has been demonstrated previously. Our studies have illustrated the protective effect of PDGF-BB on HIV Tat and cocaine caused impairment of neurogenesis by activating TRPC channels and downstream signaling pathways (Yao, Duan et al. 2012, Chao, Yang et al. 2014, Yang, Chen et al. 2015). It is also worth noting that TRPC channels also play an important role in MCP-1-mediated neuroprotection against Tat in a similar manner (Yao, Peng et al. 2009). Cocaine is a well-documented psychostimulant that increases levels of both dopamine and acetylcholine in the striatum

and affects neuronal excitability in the nucleus accumbens by regulating the trafficking of AMPA receptors (Guo, Fibuch et al. 2010, Van Dolah, Mao et al. 2011). Interestingly, it has been reported that cocaine and HIV viral protein Gp120 work synergistically to foster formation of dendritic varicosities and hamper neuronal viability via the involvement of oxidative stress, mitochondrial dysfunction and MAPK signal pathways (Yao, Allen et al. 2009).In summary, cocaine aggravates neuronal injury mediated by HIV viral proteins whereas both PDGF and MCP-1 play a neuroprotective role through a common pathway - activation of TRPC channels.

22.6   Conclusions

Although the advance of highly active antiretroviral therapy (HAART) significantly suppresses viral replication, improves the well-being of HIV patients and prolongs their expected life span, substance abuse including cocaine abuse among HIV-infected patients still poses a major challenge for health care management. Despite the fact that neurons are incapable of permissive HIV infection, the ongoing neuroinflammation mediated by activated glial cells and the presence viral proteins remains a chief cause of neuronal damage while cocaine serves as a multifactorial agent that can either work alone or synergistically with viral proteins and lead to exacerbation of HAND (Buch, Yao et al. 2012). Interestingly, both growth factors and inflammatory factors can play paradoxical roles in the pathogenesis of HAND. For instance, PDGF-BB functions as a vascular permeant in endothelial cells, but can also play a protective role in neurons against viral proteins. Similarly, chemokines such as MCP-1 function as inflammatory mediators for glial cells but also serve a neuroprotective role in neurons (Yao, Bethel-Brown et al. 2014). A better understanding of the interactions between these mediators is thus crucial for developing improved therapeutic strategies for HAND.

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23 ROLE OF SIGMA-1 RECEPTOR IN COCAINE ABUSE AND NEURODEGENERATIVE DISEASE

23.1 Overview

Drug addiction involving the use of psychostimulants such as cocaine and methamphetamine is a growing epidemic globally. Data from National Survey on Drug Use and Health (NSDUH) showed that in 2011, 670,000 American aged 12 or older had used cocaine (Abuse 2012) . It has been well documented that chronic cocaine users often suffer from symptoms such as anxiety, depression, drug craving and cognitive impairment (Aharonovich, Nunes et al. 2003, Glauser and Queen 2007). Cocaine abuse in the elderly also poses serious challenge to the health care management in the United States. In fact, one study of inner city emergency department showed that 2% of visiting patients aged over 60 years were cocaine positive based on the urine test (Rivers, Shirazi et al. 2004). Moreover, structural MRI brain scan studies have also shown that the reduction rate of global gray matter volume in the cortical and subcortical regions of chronic cocaine users was nearly twice the rate of healthy volunteers, suggesting accelerated ageing in cocaine-dependent individuals (Ersche, Jones et al. 2013). A vicious cycle thus manifests, in which cocaine users suffer from cognitive decline resulting from both cocaine abuse as well as ageing.

In addition to the mental health consequences of cocaine, increased incidence of neurodegenerative disorders is also a growing concern as individuals continue to live long. Neurodegenerative disorders, including Alzheimer's Disease (AD), Parkinson's Disease (PD), Amyotrophic Lateralizing Sclerosis (ALS) and Huntington's Disease (HD), are characterized by progressive loss of neuronal functioning and numbers. These disorders are posing a major challenge for health care management with the global increase in aging population. AD is one of the most common neurodegenerative diseases, with 46.8 million

people affected worldwide (Prince, Wimo et al. 2015). PD is the second common neurodegenerative condition, affecting around 2% of the population above age of 65 (Elbaz, Manubens-Bertran et al. 2000). The prevalence of ALS in United States is 3.9 cases per 100,000 general population, and it is more common among persons aged 60-69 years (Mehta, Antao et al. 2014). Approximately five to seven out of every 100,000 people are affected by HD in Western countries (Folstein 1989). It is therefore essential to discover novel approaches to alleviate cognitive impairment induced by cocaine and/or age-related neurological disorders.

Sigma receptors were formerly misclassified as a subtype of opioid receptor in 1970s. However, over the years their role as a unique class of non-G protein-coupled intracellular protein receptors that bind to cocaine has become more clear (Narayanan, Mesangeau et al. 2011). Sigma receptors have two subtypes, namely Sigma-1 receptor (Sig-1R) and Sigma-2 receptor (Sig-2R). Sig-2R is a universally distributed protein that modulates cell proliferation and tumor pathogenesis (Guo and Zhen 2015). Sig-1R is an intracellular molecular chaperone (28 kDa) that predominantly resides in the endoplasmic reticulum (ER), especially ER sub-region contacting mitochondria, namely the mitochondrial-associated membrane (MAM) (Mori, Hayashi et al. 2013, Nguyen, Lucke-Wold et al. 2015). Highly expressed in the brain, Sig-1R is also a transmembrane protein that regulates various cellular activities including synaptic plasticity, modulation of ion channels, ER stress, astrogliosis and microglia activation (Tsai, Pokrass et al. 2014). Recent studies have also shown that Sig-1R plays an important role in drug addiction especially cocaine abuse, psychiatric disorders and neurodegenerative disorders (Kourrich, Su et al. 2012). Understanding how Sig-1R plays a role in the pathogenesis of neurodegenerative disorders and cocaine abuse will be the subject of this review.

23.2   Role of Sigma-1 receptors in cocaine and HIV-mediated cognitive impairment

Despite the advent of combination antiretroviral therapy (cART), there is increased prevalence of HIV-associated neurocognitive disorders (HAND) (Dallasta, Pisarov et al. 1999), as infected individuals continue to live longer. Almost 40-60% of infected individuals on cART are known to suffer from some form of HAND. The key underlying correlate of HAND is inflammation, both in the periphery and in the CNS. Adding further layer of complexity to HAND is the increased abuse of drugs in those infected with HIV. Drugs of abuse further exacerbate neuroinflammation associated with HIV-1. Interestingly, one such drug - cocaine, has been shown to exacerbate neuroinflamamtion via several molecular pathways involving disruption of blood-brain barrier (BBB) integrity, astrogliosis, microgliosis and neuronal injury/death (Fiala, Eshleman et al. 2005, Yao, Allen et al. 2009, Yao, Yang et al. 2010, Yao, Duan et al. 2011, Yao, Kim et al. 2011, Yang, Yao et al. 2015). Sig-1R is known to play an essential role in cocaine-related neurologic effects. Previous studies have demonstrated that Sig-1R is localized in both the CNS and in the periphery where cocaine poses its toxic effects, and interacts with Sig-1R at physiologically relevant concentrations (Matsumoto, Nguyen et al. 2014). Cocaine acts as an agonist for Sig-1R that regulates dissociation of Sig-1R from ER chaperone binding immunoglobulin protein (BIP) (Maurice, Martin-Fardon et al. 2002, Hayashi and Su 2007). Dissociated Sig-1R translocates from mitochondrion-associated ER membrane (MAM) to the ER, plasma membrane, and nuclear membrane, regulating functions of various proteins (Maurice, Martin-Fardon et al. 2002). Recent evidence has further indicated that cocaine-mediated translocation of Sig-1R resulted in increased vascular permeability (Yao, Duan et al. 2011), impairment of the blood-brain barrier (BBB) (Yao, Kim et al. 2011), microglial activation (Yao, Yang et al. 2010, Duan, Yao et al. 2014), astrogliosis (Yao, Bethel-Brown et al. 2012, Yang, Yao et al. 2015) and neuronal injury(Yao, Allen et al. 2009, Yang, Chen et al. 2015).

Herein we discuss the role of Sig-1R in response to cocaine/HIV proteins in various cells of the CNS.

23.2.1 Blood brain barrier (BBB)

Under normal conditions the BBB functions as a highly selective permeability barrier that regulates homeostasis of the CNS microenvironment. During injury or insult following exposure to noxious drugs, BBB can be breached rendering the brain vulnerable to infectious pathogens and toxins in the circulating blood (Ballabh, Braun et al. 2004). There are extensive reports suggesting the role of Sig-1R in cocaine-mediated disruption of BBB through distinct intracellular mechanisms (Yao, Duan et al. 2011, Yao, Kim et al. 2011, Brailoiu, Deliu et al. 2015). In fact one of the reports from our groups demonstrated that exposure of human brain endothelial cells (HBMEC) to cocaine resulted in increased permeability of these cells via the activation of Sig-1R, leading in turn, to upregulation of platelet-derived growth factor-BB (PDGF-BB) (Yao, Duan et al. 2011). Detailed signaling pathway involved in this process involved sequential activation of mitogen-activated protein kinase (MAPK) and Egr-1 pathways **(Figure 23.1)**.
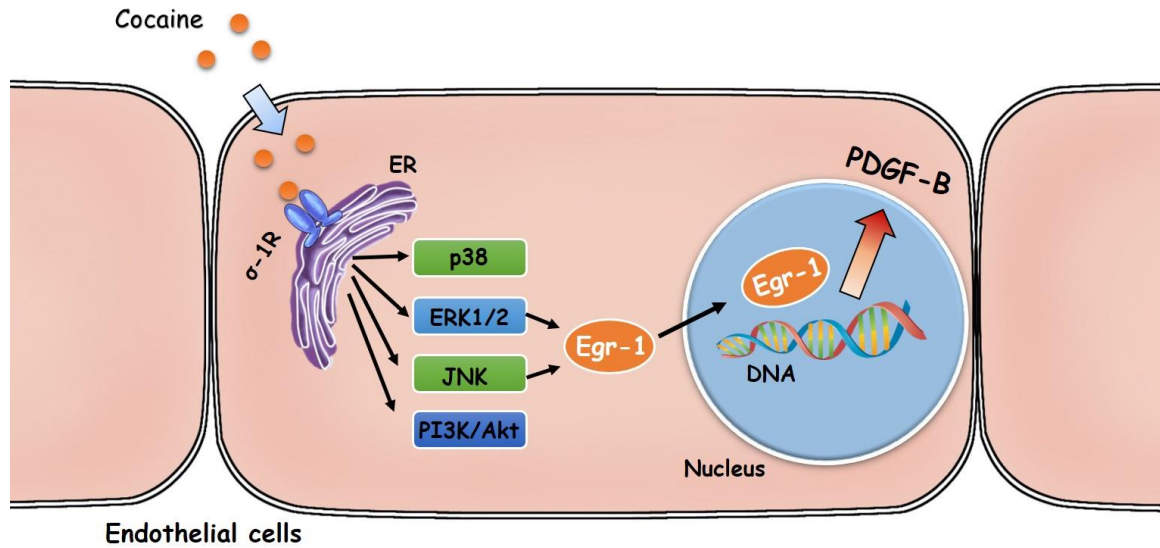
Figure 23.1 Schematic of the molecular mechanisms involved in increased permeability of HBMECs induced by cocaine.

Exposure of human brain endothelial cells (HBMEC) to cocaine resulted in increased permeability of these cells via the activation of Sig-1R, leading in turn, to upregulation of platelet-derived growth factor-BB (PDGF-BB). Detailed signaling pathway involved in this process involved sequential activation of mitogen-activated protein kinase (MAPK) and Egr-1 pathways.

These findings were also validated in vivo wherein enhanced permeability in cocaine-treated mice could be abrogated by either pre-treating the mice with neutralizing antibody for PDGF-BB or using the Egr-1 knock-out mice. Another possible mechanism for cocaine/Sig-1R-mediated endothelial dysfunction was also reported to function via inhibition of store-operated Ca_{2+} entry (SOCE) (Brailoiu, Deliu et al. 2015). In this report it was shown that cocaine inhibited SOCE in rat brain microvascular endothelial cells (RBMVEC) and, this effect could be suppressed by both an antagonist and shRNA for Sig-1R. Additionally, our group was the first to demonstrate that cocaine-mediated translocation of Sig-1R to the plasma membrane resulted in the upregulation of activated leukocyte cell adhesion molecule (ALCAM) in the HBMEC, leading subsequently, to increased monocyte adhesion/transmigration, thereby resulting in exacerbated neuroinflammation (Yao, Kim et al. 2011). Cocaine mediated induction of ALCAM involved translocation of Sig-1R to the plasma membrane, subsequent phosphorylation of PDGF-β (PDGF-β) followed by downstream activation of MAPK, Akt and NF-κB pathways **(Figure 23.2).**
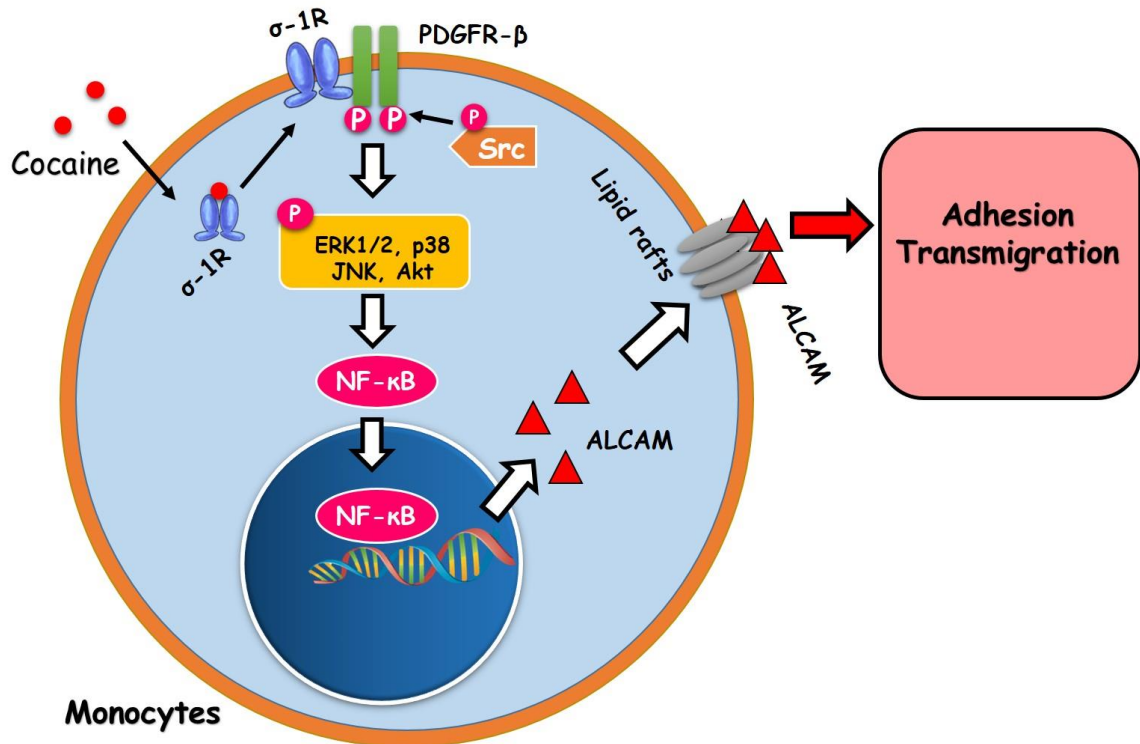
Figure 23.2 Schematic of the signaling pathways involved in cocaine-mediated induction of ALCAM.

Cocaine mediated induction of ALCAM involved translocation of Sig-1R to the plasma membrane, subsequent phosphorylation of PDGF-β (PDGF-β) followed by downstream activation of MAPK, Akt and NF-κB pathways.

Along these lines, we also observed that upregulation of ALCAM in the brain endothelium of HIV-infected cocaine users was accompanied by increased monocyte/macrophage infiltration when compared with HIV-positive individuals without drug abuse history or uninfected controls. These findings were further corroborated by the fact that neutralizing antibody to ALCAM ameliorated cocaine-mediated exacerbation of monocyte adhesion and transmigration in vitro. Understanding the regulation and functional changes of BBB by cocaine/Sig-1R axis could provide insights into the development of potential therapeutic targets for HAND.

23.2.2 Microglia

Both migration and activation of microglia/macrophages play an important role in pathogenesis of neurological disorders such as HAND. Using pharmacological inhibitors, pioneering study has indicated the involvement of both Sig-1R and TGF- β in upregulation of HIV-1 expression in microglial cell cultures in vitro (Gekker, Hu et al. 2006). Previous study from our lab has also found that translocation of the Sig-1R to the lipid raft micro-domains of the plasma membrane regulates cocaine-mediated induction of chemokine monocyte chemotactic protein-1 (MCP-1/CCL2) in microglia (Yao, Yang et al. 2010). Taking advantage of pharmacological approach, we demonstrated that cocaine-mediated upregulation of MCP-1 expression resulted from activation of Src, mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3' kinase (PI3K)/Akt and nuclear factor kappa B (NF-κB) pathways in a sequential manner.

ER stress is aberrant aggregation of misfolded proteins (Malhotra and Kaufman 2007), and prolonged ER stress often results in neuro-inflammation (Guo, Liao et al. 2015). Autophagy is a cellular adaptive mechanism in response to ER stress that delivers

misfolded proteins and damaged organelles to the lysosome for degradation (Ogata, Hino et al. 2006, Cai, Xie et al. 2014, Cai, Xie et al. 2014, Xie, Naslavsky et al. 2014, Reineke, Xie et al. 2015, Xie, Bahl et al. 2016). Accumulating evidence suggests that both ER stress and autophagy regulate neuroinflammation in various neurodegenerative disorders (Cai, Arikkath et al.). Interestingly, our recent study lends further credence to the fact that both ER stress and autophagy also play critical roles in cocaine-mediated release of inflammatory mediators in microglia (Guo, Liao et al. 2015). Upregulation of autophagy-signature mediators such as Beclin1, ATG5, LC3-II was observed in both primary rat microglial cells in vitro and cocaine-injected mice in vivo. Moreover, both antagonists for autophagy, 3-methyladenine (3-MA) and Wortmannin, successfully blocked the release of inflammatory factors in microglial cells exposed to cocaine. We also observed attenuation of cocaine-mediated autophagy in microglial cells pretreated with ER stress inhibitor salubrinal, suggesting that ER stress was upstream of autophagy induced by cocaine. Taken together, these results suggested the involvement of ER stress-autophagy axis in cocaine-mediated neuroinflammation. Targeting at both Sig-1R and ER stress-autophagy axis thus has the therapeutic potential for treating neuroinflammation in HIV-positive cocaine users.

23.2.3 Astrocytes

Astrocytes are the most abundant cell type within the brain that play various active roles in the brain, including providing structural support for BBB, maintaining the homeostasis of neurotransmitters and ions and modulating synaptic transmission (Li, Zhao et al. 2011). Toxic stimuli and traumatic insults in the CNS lead to proliferation of astrocytes and astrocytic hypertrophy with upregulation of filament protein glial fibrillary acidic protein (GFAP), known as "astrogliosis" (Colangelo, Alberghina et al. 2014). Activated astrocytes release inflammatory factors, resulting in exacerbating

neuroinflammation (Glass, Saijo et al. 2010). Accumulating evidence suggests that excessive intake of cocaine contributes to modulating the trajectory of HAND through astrogliosis (Goodkin, Shapshak et al. 1998, Fattore, Puddu et al. 2002, Yang, Yao et al. 2015). We have recently succeeded in demonstrating that Sig-1R play an essential role in cocaine-mediated astrogliosis in HAND (Yang, Yao et al. 2015). Immunostaining for GFAP in human postmortem cortex showed increased GFAP positive cells in HIV-positive cocaine users, compared with HIV-positive group without cocaine use. Both astrocytic cell line A172 and primary astrocyte culture recapitulates cocaine-mediated astrogliosis in vitro. These findings were corroborated by demonstrating upregulated GFAP in the cortex of cocaine-treated mice compared with saline injected controls. Furthermore, taking advantage of pharmacological approach, we showed that cocaine induces swift translocation of Sig-1R to plasma membrane, followed by mitogen-activated protein kinase (MAPK) signaling with subsequent downstream activation of the early growth response gene 1 (Egr-1). Activation of Egr-1, in turn, provokes transcription of GFAP. A better understanding of the cocaine/Sig-1R in mediating the astrogliosis is thus critical in dissecting the mechanism(s) underlying the disease progression of HAND and for future development of therapeutic targets.

23.2.4 Neurons

Despite the fact that direct infection of neurons by HIV-1 remains inconclusive, it has been well-known that one of the salient pathological characteristics for HAND is neuronal degeneration induced by viral proteins and virus-associated inflammatory conditions (Patel, Mukhtar et al. 2000). Accumulating evidences implicates that cocaine abuse potentiates neurotoxicity in the presence of HIV-1 viral proteins such as Gp120 (Yao, Allen et al. 2009, Yao, Bethel-Brown et al. 2009). Previous studies from our group has demonstrated the cocaine and Gp120-mediated synergistic cellular toxicity on rat

primary neurons (Yao, Allen et al. 2009). Mechanistic study has also revealed the involvement of reactive oxygen species and loss of mitochondrial membrane in the combinatorial neurotoxicity induced by cocaine and Gp120. Interestingly, mitogen-activated protein kinases (MAPK) signal pathways also plays an essential role in this process. Using pharmacological inhibitors, our group has further found that c-jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK)/MAPK pathways converges in the activation of nuclear factor (NF)-kB, leading to neuronal death. Another study from our group also reported that cocaine collaborated with Gp120 boosts dendritic beading in rat primary hippocampal neurons, ultimately culminating into the formation of dendritic varicosity (Yao, Bethel-Brown et al. 2009). In summary, cocaine potentiates neurotoxicity mediated by HIV viral proteins and determining the detailed molecular mechanism(s) will provide insights for the development of new therapeutic approaches aimed at treatment of HAND in the drug-abusing population.

23.3 Sigma-1 receptors in other neurodegenerative disorders

23.3.1 Alzheimer's Disease (AD)

AD, clinically characterized as an ongoing cognitive impairment, is the most common neurodegenerative diseases globally, with 46.8 million people affected worldwide (Prince, Wimo et al. 2015). Accumulation of neurofibrillary tangles containing hyperphosphorylated tau and Aβ plaques are two cardinal pathological features of AD (Selkoe 2001). Both postmortem and in vivo brain imaging studies have demonstrated reduced density of Sig-1R in the brains of patients with AD (Jansen, Faull et al. 1993, Mishina, Ohyama et al. 2008). Interestingly, Sig-1R density remains unchanged during physiological aging, suggesting a possible correlation between reduction of Sig-1R and

the pathogenesis of AD (van Waarde, Ramakrishnan et al. 2011). The etiology of Sig-1R loss however, remains unclear. It is known that the E4 variant of the apolipoprotein E gene (APOE 4) is a commonly recognized genetic risk factor accountable for certain fraction of late-onset AD (Bekris, Yu et al. 2010), but it remains inconclusive whether this variant is attributable to a low density of Sig-1R in AD. Interestingly, in both Australian and Chinese cohorts it has been demonstrated that the interaction of Sig-1R and APOE 4 influences AD severity (Huang, Zheng et al. 2011). In contrast, studies on cohorts of Polish and Hungarian AD patients did not show significant evidence in support of interaction between Sig-1R and APOE 4 in AD (Maruszak, Safranow et al. 2007, Fehér, Juhász et al. 2012). Further studies are required to address whether ethnicity/genetic diversity should be considered as a crucial contributing factor for the interaction between Sig-1R and APOE 4 polymorphism in the pathogenesis of AD.

Taking advantage of different Sig-1R agonists, accumulating evidence suggests a neuroprotective role of Sig-1R in AD through various mechanisms, including regulation of intracellular calcium, prevention of oxidative stress and anti-apoptotic effects. For example, pan selective Sig-1R agonist afobazole inhibits the increase of intracellular calcium level, suppresses nitric oxide (NO) production and lowers expression of the proapoptotic protein Bax and caspase-3 in rat cortical neurons exposed to amyloid beta25–35 (Aβ25–35) (Behensky, Yasny et al. 2013). Interestingly, afobazole also plays an anti-inflammatory role by decreasing microglial activation and migration and preventing apoptosis induced by Aβ25–35 in rat microglia (Behensky, Yasny et al. 2013). ANAVEX2-73, a mixed muscarinic and Sig-1R agonist, was also reported to block Tau hyperphosphorylation and Aβ1–42 production in Aβ25–35-injected mice (Lahmy, Meunier et al. 2013). In the same mouse model of AD, ANAVEX2-73 has also been shown to prevent oxidative stress and learning deficits (Villard, Espallergues et al. 2010). However, knocking down of Sig-1R

affects survival of primary hippocampal neurons and leads to degeneration (Hedskog, Pinho et al. 2013), further suggesting a neuroprotective role of Sig-1R. Clinically approved AD drug donepezil has also been demonstrated to protect memory function synergistically with Sig-1R agonists PRE-084 or ANAVEX2-73 in mice treated with Aβ25–35 (Maurice 2016). A better understanding of the role of Sig-1R in AD is thus critical in dissecting the mechanism(s) underlying disease pathogenesis and for future development of therapeutic targets.

23.3.2 Parkinson's Disease (PD)

PD is well recognized as the second most common neurodegenerative disorder characterized by bradykinesia, rigidity and resting tremors. The majority of the symptomatology of the disease are attributable to the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, resulting in the impairment of dopaminergic neurotransmission (Lang and Lozano 1998, Lang and Lozano 1998). Similar to the findings in AD, reduced Sig-1R density and dopamine release has also been observed in early PD patients using [11C]SA4503 and positron emission tomography (PET) (Mishina, Ishiwata et al. 2005). Interestingly, a link between Sig-1R and dopamine has been elegantly reported by Mori et al. in his study, it was shown that dopamine at physiological concentration (lower than 10 μM) induces apoptosis in Chinese hamster ovary (CHO) cells that were knocked down for the sigma 1-R, but not in the wildtype CHO cells (Mori, Hayashi et al. 2012). The possible mechanism underlying apoptosis involved dopamine mediated conversion of nuclear factor κB (NF-κB) p105 to the active form of p50 in the proteasome of Sig-1R knockdown CHO cells, leading in turn, to downregulation of Bcl-2 and apoptosis. Loss of Sig-1R could thus render neuronal cells vulnerable to drug-induced dopamine surge or even physiological dopamine level, resulting in turn, to dopamine toxicity (Mori, Hayashi et al. 2012). Based on these, it can be envisioned that

Sig-1R and its ligands could be developed as potential therapeutic targets for PD. PRE-084 is a selective Sig-1R agonist and has been demonstrated to restore behavioral performance as well as neuronal function in PD mouse models with intrastriatal 6-hydroxydopamine (6-OHDA) lesions (Francardo, Bez et al. 2014). PRE-084 upregulates neurotrophic factors (Brain-derived neurotrophic factor, BDNF & glial cell line-derived neurotropic factor GDNF) and their downstream, and modestly recovers dopamine levels, followed by increased density of dopaminergic fibers in striatal regions.

Levodopa is a commonly used drug for long-term treatment of PD but often accompanied by dyskinesia, which is known as levodopa-induced dyskinesia (LID). A PET study has also revealed abnormally elevated binding potentials of cerebellar sigma receptors in advanced PD patients, suggesting involvement of sigma receptors in the pathogenesis of LID (Nimura, Ando et al. 2004). It is not surprising due to the fact that Mori et al. has also demonstrated that dopamine can significantly upregulates Sig-1R expression and ER chaperone protein in CHO cells in a dose dependent manner (Mori, Hayashi et al. 2012). Sig-1R antagonist BMY-14802, previously used for treating schizophrenia, has been demonstrated to reduce abnormal involuntary movement and improve motor functions in the 6-OHDA rat model of PD through serotonin 5-HT1A receptor (Paquette, Foley et al. 2009).

Sig-1R plays an important role in maintaining the balance of dopaminergic system in the brain. Restoring the homeostasis of Sig-1R might provide insights for developing potential therapeutic targets for PD or LID. However, the intervention in the Sig-1R signaling pathway through various Sig-1R ligands should be carefully investigated because some ligands such as trishomocubanes and safinamide does not necessarily exert neuroprotective effect or improve behavioral performance through Sig-1R (Fariello 2007, van Dijk, Johnston et al. 2008).

23.3.3 Amyotrophic Lateralizing Sclerosis (ALS)

ALS is a progressive neurodegenerative disorder characterized by loss of spinal cord motor neurons (MNs), leading to weakness in the muscles and eventually death from respiratory failure (Boillee, Vande Velde et al. 2006, Robberecht and Philips 2013). It is often accompanied with other neurodegenerative diseases such as Frontotemporal Lobar Dementia (FTLD) (Ferrari, Kapogiannis et al. 2011). Annually, the prevalence of ALS in United States is 3.9 cases per 100,000 general population, and it is more prevalent among persons aged 60-69 years (Mehta, Antao et al. 2014). Although major advances been made in our understanding of the genetic causes of ALS, the pathophysiology of this disease still remains poorly understood. A number of genes have been identified and associated to the establishment of ALS including superoxide dismutase-1 protein (SOD1), RNA-binding protein Fused in Sarcoma (FUS), TAR DNA-binding protein 43 (TARDBP) and so on(Daoud, Valdmanis et al. 2009, Millecamps, Salachas et al. 2010). Under normal conditions, Sig-1R is particularly enriched in MNs present in the brain stem and spinal cord (Gundlach, Largent et al. 1986, Mavlyutov, Epstein et al. 2010); with mutations in this gene have been found to contribute to the pathogenesis of FTLD-ALS and juvenile ALS (Luty, Kwok et al. 2010, Al-Saif, Al-Mohanna et al. 2011). Prause et al. examined the expression of Sig-1R in post mortem spinal cord of ALS patients and in the SOD1 transgenic mouse model of ALS, and found presence of abnormally accumulated Sig-1R in enlarged C-terminals and endoplasmic reticulum structures of alpha MNs, which further supported the association of abnormally modified Sig-1R with ALS (Prause, Goswami et al. 2013).

Information gleaned from the studies in ALS patients and from various ALS models in the past years, has shed light on the role of mitochondrial damage and oxidative stress, excitotoxicity, neuroinflammation, ER stress, misfolded protein aggregation and defective

removal of toxic proteins as the pathological hallmarks of ALS(Rossi, Cozzolino et al. 2016), Most of these processes have been shown to be modulated by Sig-1R. For example, Mavlyutov et al. demonstrated that Sig-1R acts as a brake on MN excitability in the SOD1 G93A mouse model of ALS, while the reduced excitability may extend the longevity of MN. This is the first case wherein the absence of Sig-1R has been shown to be attributable to the shortened lifespan of ALS in the mouse model (Mavlyutov, Epstein et al. 2013). Intriguingly, Prause et.al. have also indicated that disturbances in the unfolded protein response and impaired protein degradation were related to the accumulation of Sig-1R in cultured human ALS-8 skin fibroblasts and SOD1 transgenic mouse alpha motor neurons. Furthermore in this study, deranged calcium signaling and abnormalities in ER and Golgi structures caused by shRNA knockdown of Sig-1R have also been reported to result in the apoptosis of motor neurons (Prause, Goswami et al. 2013).

Interestingly, in several published reports, Sig-1R agonist PRE084 has been implicated as a potential therapeutic strategy for neuroprotection in the ALS mouse model. For example, Mancuso et al. demonstrated that administration of PRE084 (0.25 mg/kg, i.p.) improved functioning of motor neurons and extended their survival in both female and male SOD1-G93A ALS mice (Mancuso, Olivan et al. 2012). In another study a similar therapeutic effect of PRE084 administration in SOD1-G93A ALS mice was reported and it was also shown that Sig-1R mediated neuroprotective effects on the motor neurons by reducing the number of activated astrocytes and macrophage/microglia (Peviani, Salvaneschi et al. 2014).

23.3.4 Huntington's Disease (HD)

HD, a devastating, hereditary neurodegenerative disease, affects approximately five to seven out of every 100,000 people in the Western countries (Folstein 1989). As HD is a hereditary disease, children have a 50% chance of inheriting the genetic trait from an affected parent. HD is caused by over expansion of a cytosine-adenine-guanine (CAG) trinucleotide repeat in the huntingtin gene, which is normally less than 27 repeats. CAG repeats expand through replication error to 40 or more are fully penetrant and inevitably associated with neuronal degeneration and the progressive motor, cognitive, and behavioral features of HD (Hendricks, Latourelle et al. 2009).

Normally, huntingtin shuttles between the ER and the nucleus, and plays the role in regulation of autophagy triggered by ER stress. However, mutated huntingtin loses its ability to return to the ER and starts to aggregate in the nucleus (Xia, Lee et al. 2003, Atwal, Xia et al. 2007, Atwal and Truant 2008). A recent study demonstrated that accumulation of Sig-1R is a feature common for mediating neuronal nuclear inclusions in HD, which thereby implicated the role of Sig-1R in the ER-related degradation machinery for degradation of the mutated huntingtin (Miki, Mori et al. 2014). Additionally, Hyrskyluoto et al. reported that expression of mutant huntingtin resulted in decreased Sig-1R levels in a neuronal cell line (PC6.3), which in turn, could be restored by the administration of Sig-1R agonist PRE084. These findings suggested that Sig-1R agonist PRE084 elicits beneficial effects in models of HD via positively affecting NF-kB signaling to upregulate the levels of cellular antioxidants and by decreasing ROS levels (Hyrskyluoto, Pulli et al. 2013). Furthermore, it is well known, that the dopamine stabilizer - ACR16 is in an advanced phase of clinical trials for the relief of the motor symptoms of Huntington's disease (Lundin, Dietrichs et al. 2010). This drug is thought to exert its beneficial effects primarily via the dopamine D2 receptor. Interestingly, Sahlholm et al. have provided a

novel idea that ACR16 binds Sig-1R in low concentrations, which is 100 times lower than that reported for its interaction with the D2 receptor (Sahlholm, Arhem et al. 2013). This new knowledge could be used to develop future treatments for HD. Overall, Sig-1R could be envisioned as a promising target for future drug development for HD.

23.4   Conclusions

Both in vitro and in vivo studies have significantly advanced our understanding of the molecular mechanism(s) underlying Sig-1R and have revealed the important role of Sig-1R in both neurodegenerative disorders as well as in cocaine abuse. Restoring the homeostasis of Sig-1R could provide insights for developing potential therapeutic targets for neurodegeneration and for cocaine-related neurologic impairments. It must be cautioned however, that the molecular regulation of Sig-1R pathways still remains to be elucidated in detail. The intervention in the Sig-1R signaling pathway by various Sig-1R ligands should be carefully investigated because some ligands with high affinity do not necessarily exert neuroprotective effect or improve behavioral performance through Sig-1R (Fariello 2007, van Dijk, Johnston et al. 2008).

CHAPTER XI

Summary

Drug abuse among HIV-infected patients poses a major challenge for health care management and one of the hallmark features of opiate abuse is the increased neuronal toxicity in the setting of HIV infection (Hauser, Fitting et al. 2012). Opiate abuse and HIV-1 have been described as two linked global health crises. While the advances in antiretroviral therapy have transformed the conception of HIV/AIDS from a death sentence to a manageable chronic condition , co-morbid condition of opiate abuse in infected individuals is on a rise, leading in turn, to increased neurologic & cognitive deficits, collectively termed as HIV-associated neurocognitive disorders (HAND) (Cherner, Masliah et al. 2002, Diesing, Swindells et al. 2002, Lawrence and Major 2002, Gonzalez-Scarano and Martin-Garcia 2005). Intriguingly, brain regions that are targets for morphine (expressing high numbers of μ-opioid receptors), such as the basal ganglia & the hippocampus, co-incidentally are also the regions with increased viral loads and predilection for HIV-1 (Foley, Ettenhofer et al. 2008). Therefore, the CNS may be uniquely susceptible to the combined effects of opiate abuse and HIV-1.

Morphine and related opioids are widely used in the clinical setting resulting in beneficial analgesic effects. However, prolonged use of morphine is also associated with deleterious side effects such as opioid tolerance, dependence, addiction and cognitive decline (Mao, Price et al. 1995, Kosten and George 2002, Hyman 2005, Fields and Margolis 2015). Morphine use has been known to interfere with learning and memory processes (Kerr, Hill et al. 1991, Hill and Zacny 2000, Skaer 2004, Bodnar and Klein 2005, Tan, Duan et al. 2015, Hearing, Jedynak et al. 2016). Learning and memory are intimately linked to the structure, function and plasticity of synapses (Caroni, Donato et al. 2012, Josselyn, Kohler et al. 2015, Smolen, Zhang et al. 2016). While the majority of studies have demonstrated significant reduction in dendritic spine density in the nucleus accumbens, hippocampus and neocortex of adult rats with morphine (Robinson, Gorny et

al. 2002, Miller, Zhang et al. 2012), our understanding of the molecular basis of how morphine leads to the cellular alterations that contribute to cognitive decline is scant (Robinson, Gorny et al. 2002, Marder and Goaillard 2006, Zhong and Gerges 2010, Mazei-Robison, Koo et al. 2011, Sadegh and Fathollahi 2014, Muller, Quednow et al. 2016), thus limiting options to provide effective therapeutic interventions to ameliorate cognitive decline associated with long-term morphine use. Our studies identify a novel linear pathway linking morphine mediated activation of the MOR in neurons to alterations in excitatory and inhibitory synapse densities in the hippocampus, thus providing a novel mechanism for morphine induced cognitive changes. More importantly, we provide key evidence that morphine induced synaptic alterations can be prevented by treating neurons with PDGF-BB, a robust pleiotropic and broad-acting neuroprotective agent (Peng, Dhillon et al. 2008, Peng, Dhillon et al. 2008, Zhu, Yao et al. 2009, Keller, Westenberger et al. 2013, Johnson, DeKorver et al. 2014, Yang, Chen et al. 2015). These studies thus underscore a molecular mechanism for cognitive changes associated with morphine use and a strategy for ameliorating these deficits.

Neurocognitive complications of HIV are associated with reduced quality of life, memory loss, and social & occupational disabilities (Cherner, Masliah et al. 2002, Diesing, Swindells et al. 2002, Lawrence and Major 2002, Gonzalez-Scarano and Martin-Garcia 2005). Development of therapeutic strategies that can reverse the cognitive decline and the associated pathology are thus an urgent need. By carefully selecting specific antiretrovirals and supplementing them with neuroprotective agents such as PDGF, physicians might be able to promote CNS repair, thereby facilitating enhanced neural function and clinical neurological status.

In the proposed project, we will examine the role of morphine (one of the opiate drugs that are commonly used in HIV positive patients) in inducing synaptodendritic

alterations in neurons. Additionally, we will also test the role of the neurotropic factor PDGF in reversing this effects.

Our data provide evidence that morphine-mediated activation of the mu opioid receptor (MOR) in hippocampal neurons results in the sequential generation of ROS, ER stress and autophagy, leading to a decrease in the density of excitatory synapses and increase in the density of inhibitory synapses **(Figure 1)**. Morphine mediated synaptic alterations could be reversed by PDGF-BB. The protective effects of PDGF-BB are mediated at the level upstream of ROS generation and by preventing the activation of downstream ER stress and autophagy pathways.

Our data indicates that treatment of morphine-exposed neurons with PDGF-BB ameliorated morphine-mediated synaptic alterations in hippocampal neurons. This effect of PDGF-BB was at the level of inhibition of ROS production following activation of MOR, leading in turn, to inhibition of downstream ER stress and autophagy pathways, ultimately leading to preservation of excitatory and inhibitory synapses.

Thus, our results strongly support a model in which morphine exposure leads to sequential generation of ROS, initiation of ER stress and enhanced autophagic activities to modulate both excitatory and inhibitory synaptic densities in the hippocampal neurons. Furthermore, we provide a novel neuroprotective role of PDGF-BB in blocking morphine-mediated synaptic alterations. It can thus be envisioned that therapeutic strategies aimed at activating PDGF-BB signaling could be beneficial in reversing morphine-mediated cognitive decline. These findings could have clinical ramifications in the future development of therapeutics aimed at managing cognitive decline in chronic morphine users as well as HIV infected opiate abusers.

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